Factors Influencing the Susceptibility of Apple Trees to *Trametes* (*Polystictus*) *versicolor*

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person except where due reference is made in the text of the thesis.

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Abstract

"Polystictus dieback" of apple trees is a serious problem in Australian orchards and the literature suggests that the disease is only extensive and causes severe injury to trees in orcharding areas of this country. The disease is associated with a wood rotting Basidiomycete, Trametes versicolor, and as a result of attack by this fungus limbs or whole trees may be destroyed. By growing apple trees in sand culture a quantitative relationship was established between a phosphorus deficiency and an increased susceptibility of limbs to destruction by the fungus (Wade, unpubl.).

The results reported here are directed towards an attempt to explain the parasitic nature of T. versicolor on apple trees in Australia. A field survey failed on confirm any relationship between phosphorus status and the incidence of dieback in trees.

The effects of reserve nutrients accumulated by trees on the development of the fungus were then examined using in vitro methods. The fungus was grown on blocks of apple wood in culture jars containing different nutrient sources incorporated in a medium. When a carbon source was absent from the media, decay of wood was high. With a high carbon

or nitrogen deficient media wood decay was comparatively low. Solution culture experiments indicated that production of extracellular polyphenol oxidase is an important factor in this effect. With increasing glucose content of media, decay of wood was increasingly suppressed. Polyphenol oxidase production was suppressed with increasing media sugar levels.

By extracting wood blocks before subjecting them to attack by the fungus the presence in the wood of an inhibitor to the growth of the fungus, was demonstrated. It was postulated that such an inhibitor may be associated with soluble sugar levels in trees.

Inoculation trials demonstrated a varietal variation in susceptibility of trees to the disease, an increased amount of dieback resulting from winter inoculations and an inability of the fungus to grow in one year old wood. The trials also indicated a relation between high soluble sugar levels and a lower rate of establishment of the fungus. This result although not entirely conclusive, confirmed the results of the in vitro experiments.

It is concluded that environmental and managerial conditions to which trees are subjected in Australia, result in

an increased susceptibility of limbs to attack and ultimate destruction by T. versicolor. The increased susceptibility of branches may be explained in terms of a lowered soluble sugar content and an associated lower level of the suggested inhibitory factor to the growth of the fungus.

GENERAL INTRODUCTION

"Polystictus dieback" or "papery bark" of apple trees is a common problem in Australian orchards. Symptoms are recognizable by the presence of dead limbs in association with a blistering and peeling off of bark (see Figures 1 and 2). The disease can affect a portion of a limb or the whole tree may be destroyed. A wood rotting Basidiomycete, Trametes versicolor (Linnaeus ex Fries) Lloyd[+], can virtually always be isolated from limbs displaying the described symptoms.

Outside Australia the occurrence of the disease has been reported only in isolated cases. Brooks (1923) mentioned considerable damage to apple trees in the English fruit-growing district Wisbech, by the "entry of a fungus through wounds which were made in the process of thinning out mature trees, large wounds being particularly prone". Generally, however, T. versicolor is considered to be merely a saprophyte in Britain (Wormald, 1955).

In America, Smith (1930) demonstrated that in Eastern Colorado Trametes hispida was frequently found on apple trees and on this host it functioned primarily as a parasite. Eide and Christensen (1940) expressed the opinion that decay by

[+]This fungus has been named according to Cunningham (1965). Ainsworth (Comm.Myc.Inst., Kew) (1961) uses the generic name "Polystictus".

FIGURE 1

1A (above) - "Polystictus dieback" - Illustrating dead limbs on an apple tree as a result of infection by Trametes versicolor.

1B (below) - Final effect on a tree following infection by T. versicolor. A .303 cartridge case has been hammered into the crotch of the tree - a method used (unsuccessfully) in an attempt to prevent the extension of dieback of a limb.

FIGURE 2

Dead limbs, infected with T. versicolor, displaying the typical papery bark symptoms. The white mycelium of the fungus can be seen in the broken base of the branch on the right.

wood rotting fungi may be one of the causes of the early decline of apple trees in marginal fruit growing areas in Minnesota.

Records from Africa and India quoted by Eide and Christensen (1940) demonstrate the presence of the problem in those countries. T. versicolor has also been isolated from cherry and apple "twigs and branches with a white pith" from orchards in Switzerland (Blumer and Nuesch, 1962).

However, the general impression given by the literature is that T. versicolor is not an important pathogen of apple trees in countries outside Australia.

Doepel (1962, 1965) reports the serious and persistent nature of the disease in Western Australian orchards for many years. Anon. (1956) records the presence of wood rotting fungi and their association with an annual toll of health and productivity of fruit trees in New South Wales. In Victoria, Baxter (1957a, 1957b) mentions the presence of dieback in orcharding areas. Ward (1957) reports the need in Tasmania of proper orchard care to reduce the incidence of dieback.

It is presumed that the fungus enters a branch (from air-borne spores) through wood that has been exposed to the environment as a result of leader decline, pruning and

grafting wounds, sun burn, implement damage or mechanical damage due to fruit load.

Doepel (1965) mentions that the interaction of dieback and overcropping has been observed and investigations have "highlighted the reduction of top growth" brought about by overcropping. The result of overcropping has been shown by Mochizuki (1962) to result in a lowered carbohydrate concentration in the tree as a whole. This could be expected to bring about a reduction in growth and if practised over a period of time, the gradual decline of tree vigour. The same effect may be ascribed to hard pruning and factors resulting in the damage of roots such as deep cultivation, drought and water logging conditions.

Mineral deficiencies are also known to be associated with a greater occurrence of dieback. Copper (Dunne, 1938), manganese (Dunne and Gulvin, 1946) and zinc deficiencies (Wade, 1949) have been recorded as being associated with general tree decline and dieback. Baxter (1957a, 1957b) has shown that potassium fertilization generally increased tree vigour and resulted in a lowered incidence of decline and dieback in orchard trees.

Apple trees grown in sand culture under phosphorus deficient conditions have been shown to display a marked increase in susceptibility to attack by T. versicolor (Wade,

unpubl.). This was in contrast to similar trees grown under complete nutrient conditions where, although the fungal mycelium penetrated the branch, the external symptoms did not proceed rapidly (see Table 1).

Control measures generally involve improved cultural and management methods. Doepel (1965) suggested that adequate irrigation should be used wherever possible coupled with lighter pruning and prevention of overcropping as essential basic practices to be adopted. All wounds should be sealed with paint containing copper oxychloride and orchard hygiene, in the form of burning all prunings and infected wood to minimise fruit body formation, are necessary to reduce the risk of infection (Ward, 1957).

It is known that tree decline as a result of environmental and managerial influences tend to increase the risk of infection. Actual physiological changes occurring in the tree, as a result of decline in vigour and giving rise to an increased susceptibility to attack by T. versicolor, remain to be elucidated.

An explanation of the parasitic nature of T. versicolor in apple trees is of particular relevance to the Australian orcharding industry. Production losses result from the destruction of limbs and whole trees following infection by the fungus.

Table 1 (from Wade, unpublished)

Effect of mineral nutrition on development of dieback symptoms in limbs of trees inoculated with T. versicolor.

Nutrient Treatment		Length of External Symptoms in mm. 5 Months After Inoculation	Penetration of Hyphae in mm.	Phosphorus as % of dry weight Leaves	Wood
Complete	1	0	40	.12	.014
	2	0	30	.10	.014
	3	0	50	.14	.015
	4	0	30	.14	.011
	5	0	50	.16	.016
	6	0	105	.10	.036
Minus Phosphorus	1	80	140	.07	.008
	2	250	250	.10	.002
	3	20	40	.08	.009
	4	75	80	.09	.004
	5	45	80	.08	.004
	6	65	110	.08	.006

Because of the commercial importance of the effects of this disease and lack of knowledge regarding its development, the investigations reported in this thesis were directed towards explaining the parasitic nature of T. versicolor on apple trees.

PREFACE

The investigations reported in this thesis commenced with a field survey of orchard trees considering environmental and managerial factors that may give rise to an increased susceptibility of trees to attack by Trametes versicolor. The factors investigated included water logging, mineral status and orchard management with respect to the use of irrigation, fertilizers and general orchard hygiene. Particular attention was directed towards attempting to establish a field relation between the phosphorus nutrition of orchard trees and the incidence of dieback but levels of potassium and calcium were also estimated. This was done in an attempt to show whether the results obtained in sand culture experiments (Wade, unpubl.) could be extended to the field situation. In Wade's work limbs of phosphorus deficient trees were shown to be much more susceptible to destruction following inoculation with T. versicolor than limbs of trees receiving a complete nutrient treatment. As these results suggested phosphorus nutrition may have been an important factor influencing susceptibility of apple trees to dieback the movement and distribution of this element in the wood of limbs was examined using radioactive phosphorus. Chapter 1 records the results of these investigations.

After failing to establish a relationship between the mineral nutrition and incidence of dieback in field trees it was decided to examine the influence of reserve nutrients (carbohydrates and nitrogen) in the development of decay of apple wood by T. versicolor. In vitro experiments demonstrated that increasing glucose levels suppressed the amount of wood decay. Chapter 2 contains details of this work which supports the idea of Polystictus dieback of apple trees being a low sugar disease and contains evidence for the presence of an inhibitor to fungal growth, being contained within the methanolic extractable fraction of apple wood.

In Chapter 3 the effects of inoculating orchard trees with T. versicolor at different times of the year and wood of different ages is recorded. Carbohydrate levels of the wood at the time of inoculation, were estimated in an attempt to relate these levels with the extent of external dieback symptoms that developed.

CHAPTER 1

Aspects of the Phosphorus Nutrition of Apple Trees in Relation to the Incidence of "Polystictus Dieback".

I. INTRODUCTION

Because a low phosphorus status had been quantitatively associated with an increased susceptibility of trees to attack by T. versicolor (General Introduction), it was decided to extend these investigations by:

(i) attempting to establish such a relation in field trees. In addition to phosphorus, it was decided to determine potassium and calcium levels to see if these elements were implicated in the appearance of "Polystictus dieback" in orchard trees.

(ii) trace the movement of phosphorus, using radioactive phosphorus, in the wood of trees.

A review of literature pertaining to the phosphorus nutrition of apple trees is included in the present Chapter of this thesis, followed by the results and conclusions from the two proposed avenues of investigation mentioned above.

II - LITERATURE REVIEW

The Phosphorus Nutrition of Apple Trees.

Introduction

Phosphorus plays a fundamental role in the growth and survival of living cells. It is closely concerned with many vital growth processes in plants, being a component of nucleoproteins and a large number of enzymes involved in reactions dependent upon phosphorylation. Wallace (1961) points out that phosphorus is required in many biochemical reactions concerned with the metabolism of fats, carbohydrates and proteins in which phosphorylated compounds act as intermediaries.

It is therefore a constituent of the cell nucleus, essential for cell division and for the development of meristem tissue. Phosphorus concentrations in these tissues can easily be demonstrated by the use of radioactive phosphorus (P^{32}) and of radioautographic technique (Russell, 1961). Phosphorus is of importance in the germination of seeds, metabolism of seedlings and development of roots. It also functions as a buffer to maintain satisfactory conditions of acidity and alkalinity in plant cells and acts in the efficient function and utilization of nitrogen.

Deficiency Symptoms

Considering the functions of phosphorus, it is understandable that symptoms will develop when plants are grown in environments deficient in this element. With respect to apple trees, Davis (1929) and Davis and Hill (1941) have noted the following conditions that are likely to occur. Shoot growth is slender and petioles of the leaves are somewhat upright. The leaves may be smaller than normal and they tend to be dark green with reddish or purplish tinting of the midrib and larger veins. In addition to an abnormally dark green colour, the leaves may take on a bronzed appearance in the interveinal regions. There may be delayed bud burst in the spring with few lateral buds developing. Due to the importance of phosphorus in the efficient function and utilization of nitrogen, several important deficiency symptoms are similar to those resulting from a deficiency of nitrogen.

The effect of phosphorus deficiency on fruiting is an earlier ripening than normal and a tendency for internal browning to develop soon after maturity (Chandler, 1957). Evidence also suggests a correlation between firmness and phosphorus content of the foliage (Eggert, 1961)

Responses to Applied Phosphates

It is estimated that a mature apple tree requires approximately 0.22 Kg. of phosphorus per year, half of which is utilized in the leaves and fruit and the remainder in the growth of tops and roots (Heinicke, 1937). Despite this and the obvious importance of phosphorus in tree nutrition, responses to the addition of this element to trees in the field have rarely been reported. Some effect in growth of one year old apple trees has been observed, but no further responses occurred after the first year (Lilleland, 1936, and Lilleland et al., 1942). Control trees, unfertilized with phosphates, produced normal growth and fruited as well as heavily fertilized trees. In the same location annual crops failed to make satisfactory growth on unfertilized plots. More recently extreme uniformity has been found in the phosphorus level in apple tree tissue regardless of fertilizer treatment (Batjer, et al., 1952).

Phosphorus requirements of apple trees are then apparently very low. Work with the variety York Imperial grown under varying concentrations of nitrogen, phosphorus and potassium illustrates this. Concentrations of nitrogen varied from 0-168 p.p.m. in the nutrient solution and it was observed that below 60 p.p.m. growth was reduced.

Potassium concentration varied from 0-117 p.p.m. and below 60 p.p.m. growth was reduced but not as much as in the minus nitrogen treatment. However in phosphorus treatments, the levels of which ranged from 0-93 p.p.m., growth was uniform in all plants receiving 4 p.p.m. of phosphorus or more, and deficiency symptoms only occurred when phosphorus was completely lacking (Batjer and Degman, 1940). Likewise Cullinan and Batjer (1943) found that trees did not respond in growth to increments beyond about 4 p.p.m. and Mochizuki and Hanada (1958) reported that trees showed a slight growth decline at 4 p.p.m. but no deficiency symptoms.

Mean absorption rates of nitrogen, phosphorus and potassium have been suggested to be 100:20.9:65.0 in a year of low yield, and 100:10.9:85.2 in the following year when a large amount of fruit was harvested (Mori and Yamazaki, 1955, and Yamazaki and Mori, 1958). This would indicate the relatively larger amounts of nitrogen and potassium required for fruiting without a complementary increment in the requirement for phosphorus. Ratios of absorption in non-bearing trees were given as 100:13.3:46.3, indicating a drop in the potassium utilized as compared with nitrogen, but with little change in phosphorus utilization. These ratios were calculated by measuring the amounts of the three elements absorbed and not the quantities present

in vegetative parts.

The lack of response to phosphate application has been attributed to the efficiency of apple trees in taking up phosphorus from soils of a low phosphorus status (Batjer et al., 1952).

With respect to foliar applied phosphates, the absorption of a range of P^{32} labelled salts by the plant leaf has been tested. Di-ammonium phosphate is more readily absorbed than mono-ammonium, di-sodium, tri-sodium and mono-calcium phosphate (Eggert et al., 1952 and Koontz and Biddulph, 1957). Increasing amounts of P^{32} were found in the leaves above the point of application. Phosphorus sprays then, when applied to leaves and branches, can be absorbed and translocated to other parts of the tree. However phosphorus applied to young leaves will not be mobilized till the leaf has matured (Koontz and Biddulph, 1957), when the element will be withdrawn to support new growth or be transported to reserve sites.

Three to five sprays of di-ammonium phosphate appeared to improve growth in minus phosphorus cultures (Eggert and Kardos, 1954), but variation in growth of replicate trees resulted in variations in total terminal growth which made it impossible to demonstrate significant differences. The

absorption of foliar applied phosphorus was decreased by adding phosphorus to the substrate and increased by increasing the number of sprays.

From the responses obtained, Eggert and Kardos (1954) concluded that foliar applied phosphorus was unable to supply adequately all the needs of the trees. It is possible however, that trees receiving sub-optimal supplies of phosphorus from root media, may benefit from foliar applications of the nutrient.

Effects of Interactions of other Elements, Water, and the Presence of a Pathogen on Phosphorus Metabolism within an Apple Tree.

There are a number of reports regarding interactions of phosphorus with other elements. Perhaps the most striking is that involving nitrogen. Increased rates of inorganic nitrogen often cause a serious reduction in the phosphorus content of leaves (Weeks et al., 1952). This is almost certainly due to the demand on phosphorus to support rapid new growth initiated by high nitrogen levels.

Baroccio (1962) reports that sodium will increase the uptake of phosphorus, gypsum will lower total phosphates

in plants, while humic acid sulphates assist in phosphorus absorption. Baroccio also pointed out, that while calcium favours the formation of calcium phosphates in the soil, magnesium will increase their absorption. Boron when applied in small quantities can stimulate phosphorus uptake (Stankovic and Becarevic, 1955).

Lack of water will detrimentally effect the ability of plants to take up both potassium and phosphorus from the soil (Hibbard and Nour, 1959). An adequate supply of moisture is necessary to maintain the levels of these elements in the leaves.

Stankovic and Becarevic (1955) showed that the presence of a pathogen in some cases alter the metabolism of phosphorus in trees. Clasterosporium carpophillum was shown by these workers to cause an increased metabolism in the leaves of sweet cherry, but a reduction in metabolism in wood. They also showed that Venturia inequalis had similar effects in apple trees.

Seasonal Effects on Translocation of Phosphorus in the Tree

Seasonal changes have a marked effect on the quantities of total phosphorus in the various tissues of trees. For fuller understanding of these changes it is useful to

mention first the growth phases of the apple tree through the course of a growing season.

First, in Spring, there is a period of formation of new tissue, the materials for which are drawn from the reserves in the tree, laid down during previous years. Secondly the new leaves are able to contribute photo-synthesized compounds from raw materials being currently absorbed through the leaf and root surfaces. These newly formed compounds are translocated to support the formation and expansion of further new tissue. Finally after cessation of growth, compounds of the nutrient elements are redistributed to reserve "sinks".

In examining whole trees Mason and Whitfield (1960), found that phosphorus concentrations in leaves and blossoms were relatively high at the beginning of the season, and then fell to a stable figure. Phosphorus in the bark exhibited a sharp fall at the beginning of the season, in support of early leaf development. Towards the end of the season levels in the bark and branches rose to 0.13% (on a dry weight basis) which was comparable with that at the beginning of the season. Precisely the same pattern was followed in the wood of the previous season, where levels fell from 0.08% D.W. early in the season and rose to this

figure again at leaf fall. This fluctuation is seen to a lesser degree in the branch wood and is not evident in the wood of trunks.

In non-bearing trees Mori and Yamazaki (1955) found that the absorption curve for phosphorus was analagous to that of nitrogen, except in terms of absolute amounts. Absorption increased gradually in parallel with the rising of atmospheric temperature and growth increase, and reached its maximum at the time of highest temperature. Absorption then decreased gradually with decreasing temperature.

In bearing trees the seasonal absorption of phosphorus was similar to the absorption in non-bearing trees, but the peak of absorption occurred later (Mori and Yamazaki, 1955).

Detailed work by Mochizuki (1964) on the distribution of radioactive phosphorus (P^{32}) following its application at various times throughout the year (June, July, September and October), revealed that the element initially accumulated in the small roots and leaves but later became redistributed as plant growth proceeded. The pattern of redistribution varied with the growth stage at which the isotope was applied. P^{32} tended to accumulate in the parts

of the tree which were growing most vigorously at the time of application. P^{32} applied in June accumulated most markedly in the fine roots and leaves. In July and September a decline in the growth of leaves and twigs resulted in a lowered rate of accumulation of phosphorus in these parts. Compensating for this a greater accumulation in the trunks and old roots occurred indicating increased growth in these regions. In November 75% of the phosphorus applied in October remained in the root system, compared with 40% remaining in November from the September application and 30% in October from the July application.

Nutrition of individual roots and their subsequent supply of nutrients to stem sections is of some interest. Movement of nutrients from one root into another occurs only if there is a considerable difference in concentration between the two roots (Kolesnikov and Palkevi, 1963). Phosphorus movement into leaves of a specific branch can be related to individual roots that virtually act as the sole supplier of the element to that branch.

Perhaps one of the main points of interest regarding phosphorus nutrition of the apple tree is the general lack of response to phosphorus fertilization. Reasons for this

appear to be closely related to its withdrawal from the leaves and accumulation in the bark, which has been shown to occur towards the end of the growing season. This reserve is then used to support early growth in the following Spring. Hence a general reutilization of phosphorus is implied and it is on this point that differentiation between the annual and perennial plant is made. Finally, phosphorus when taken up at any time of the year, will first appear in parts growing most actively. This is to be expected considering the important role this element plays in the formation of new tissue.

III - The Incidence of "Polystictus dieback" in Relation to the Phosphorus Status of Apple Trees in Southern Tasmania.

Introduction

Mineral deficiencies have been observed to be associated with Polystictus dieback of apple trees. Wade (unpubl.) demonstrated quantitatively, by growing apple trees in sand culture, that a phosphorus deficiency increased susceptibility of trees to attack by T. versicolor. Trees grown in sand culture under complete nutrient conditions did not display symptoms although hyphal penetration of inoculated branches occurred (see Table 1, p.13).

The first part of the investigations reported in this thesis involved attempting to establish a field relation between the incidence of dieback and a low phosphorus status of orchard trees. To do this a survey of some orchards in the Huon Valley (the major apple growing area in Tasmania) was conducted.

Description of the Survey Area

The Huon Valley is situated approximately twenty miles S.W. of Hobart. The average rainfall of the area is 35 inches. The soils of the Huon Valley have been described in some detail by Stephens (1935). With the

exception of alluvial soils, all are of a podzolic nature and the "differentiation into types is based on the soil texture and the horizon succession of the profile which, except for some light subsoil and hardpan phases is of a remarkably rigid form". (Stephens, 1935). Stephens (1935) demonstrated that trees growing with most vigour grew on soils containing highest levels of both potassium and phosphorus (K 0.147%, P 0.015%) while trees showing poor growth were located on soils showing lower levels of both these elements (K 0.024%, P 0.012%). Analytical data suggested the use of phosphatic fertilizer but this need was not justified according to field observation of tree condition.

Both the phosphorus and potassium status of soils mentioned above in the case of poor tree condition, were of interest in relation to tree decline and dieback. Baxter (1957a, 1957b) has shown a relationship between potassium fertilization and an increase in tree vigour and a reduction in the incidence of dieback. Wade (unpubl.) demonstrated the importance of adequate phosphorus levels of trees grown in sand culture to reduce the extent of dieback.

Survey Procedures

The following survey procedures were adopted:

(a) Approximately fifty orchards were sampled, making the survey comparatively extensive.

(b) Thirty trees were sampled from each orchard including fifteen infected and fifteen uninfected trees.

(c) Sufficient current seasons growth was taken from each sample tree to carry out phosphorus, potassium and calcium analysis of the wood.

(d) For each infected sample tree infection was rated according to severity in the following manner,

Rating	Severity	Extent
1	Slight	6" and less
2	Moderate	6-18"
3	Severe	Over 18"

An overall value for the degree of infection was calculated for each tree by multiplying the number of branches that were slightly infected by 2, those moderately infected by 3 and those severely infected by 4.

Example					Overall Rating
5 branches	slightly infected	- denoted	5(1)	- 5 x 2 =	10
3 "	moderately "	- "	3(2)	- 3 x 3 =	9
1 "	severely "	- "	1(3)	- 1 x 4 =	4
				Total	23

Uninfected trees were given the value of unity. Thus an overall rating for each tree according to the amount of infection was obtained.

When carrying out the survey the most difficult problem involved the assignment of the infection rating. First it had to be decided if the symptoms displayed by a dead limb were consistent with those caused by T. versicolor. To assist this a considerable amount of dead wood was sampled and procedures for isolating the fungus undertaken. This was done to confirm the visual appraisal of whether a limb was in fact continuing to dieback because of fungal infection.

Cultural practices of some orchardists also added to the difficulty of assigning the infection rating. For example the partial removal of a dead limb gave the impression of a lower incidence of infection. A large, healed pruning wound could be interpreted as being the result of

removal because of dieback, mechanical injury or normal pruning. In addition it was difficult to assign a rating to limbs which had been partly removed, but continued to die back.

Despite the difficulty involved in the assignment of a rating it is believed that sufficient precision was maintained to obtain relations, should they exist, between the incidence of infection and the estimated variables.

Sample Preparation

Current seasons growth taken from each sample tree was recorded according to orchard number and sample number within the orchard. As the wood was to be analysed, following each day's sampling the bark, wood and leaves were separated. (The bark becomes almost inseparable from the wood if this step is not performed almost immediately after removal from the tree.) The wood was dried before being ground in a Wiley Mill. Each ground wood sample was then transferred to a numbered glass vial and oven dried at 75°C for three days.

Chemical Analysis

A description of the methods used for the estimation

of phosphorus, potassium and calcium is given in Appendix 1. Samples were analysed in duplicate and results expressed in terms of percent dry weight. A total of approximately 560 samples were analysed which included the samples collected from eighteen of the orchards surveyed.

Statistical Analysis

The effect of phosphorus, potassium and calcium levels on the incidence of dieback was determined by the estimation of regression equations. Included in the regression equations were the ratios of the three elements (P/K, P/Ca and K/Ca). Thus from each orchard from which samples were analysed a multiple regression equation having six variables was calculated. (See Appendix IIA). The significance of the regression coefficients in each of the regression equations from eleven orchards was calculated. This was done by means of a "t" test and is tabulated in Appendix IIB. In addition correlation coefficients between each of the elements and the extent of dieback were calculated (see Appendix IIC). All of the above calculations were done using an Elliott 503 Computer installed at this University.

Average levels of phosphorus, potassium and calcium and the average ratio between the elements were estimated

for each orchard (Appendix IID). Analysis of variance were done to determine if any difference existed between orchards with respect to the phosphorus, potassium and calcium levels of trees (Appendix IIE).

Results

It can be seen from Appendix IIB that no significant relationship exists between the incidence of dieback and phosphorus levels in orchard trees. Potassium and calcium levels were also shown not to be related to the disease.

Phosphorus, potassium and calcium levels differed significantly between orchards with greatest variation in potassium levels (Appendix IIE).

Discussion

Despite previously reported results no relation could be established between dieback and the phosphorus, potassium and calcium nutrition of apple trees. This suggests that mineral element deficiencies may act indirectly in increasing the susceptibility of trees to attack by *T. versicolor*.

IV - The Distribution of Phosphorus in the Wood of Apple Trees

Introduction

In addition to assessing the incidence of Polystictus dieback in relation to the phosphorus status of apple trees, the movement and distribution of this element was traced in the wood of tree limbs. This wasdone as the second part of applying the results of sand culture experiments to field conditions in attempting to ascertain why phosphorus deficient apple trees displayed a marked increase in susceptibility to attack by T. versicolor.

Methods

Only qualitative methods using radioactive phosphorus (P^{32}) and autoradiography were employed. P^{32} was injected into the branch of a tree and its movement traced by inserting and exposing X-ray plates in the branch and later sectioning the branch and exposing plates to the sections.

Injection Method

Two $\frac{3}{8}$" holes were drilled (so as to be apposed to each other at an angle of 90 degrees) approximately three quarters of the way through the base of the branch. Rubber bungs (1 7/8" diam. cut to $\frac{3}{8}$" thickness), with a hole

drilled through the centre to receive a length of polythene tubing, were strapped (using rubber tubing) over the two holes previously drilled in the branch. Distilled water was used to commence a stream into the tree. P^{32}, in two containers each holding 10 mc. of active component in 750 mls. of inert 0.01 M. Na_2HPO_4, was transferred to Sterimag plastic bags which were slung in the tree above the two drilled holes. The tubes (connected to the rubber bungs) were then introduced into the bags and the contents allowed to drain into the branch.

Longitudinal Slits

Five slits (each 5" long and approximately 1/16" wide - to receive a 7" x 5" Kodirex Medical X-ray Plate) were cut through the branch using a coping saw. The relative positions of the slits are seen in Figure 3. Plates were exposed for 30 minutes before being removed and developed.

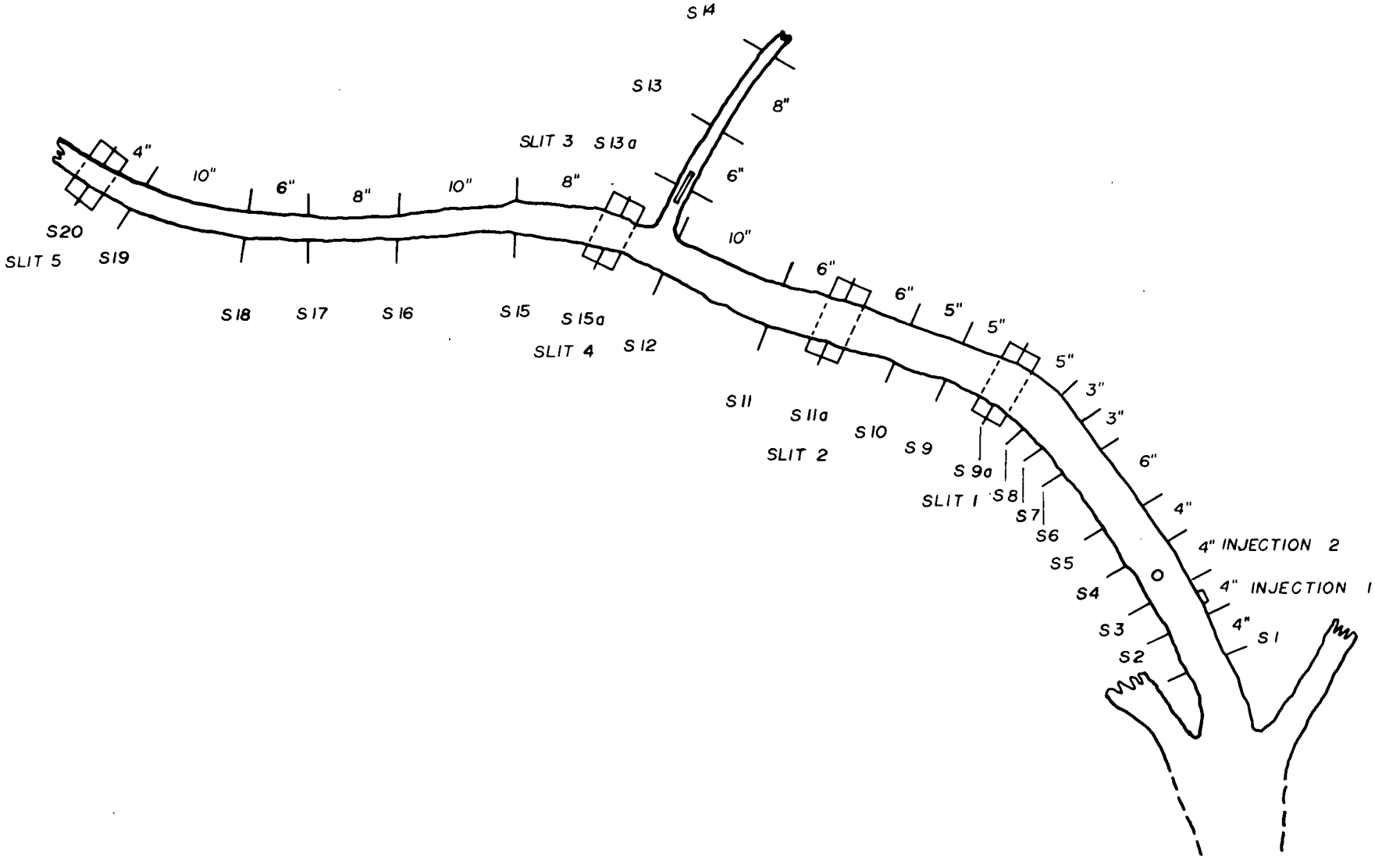
Transverse Sections

A number of transverse sections were cut at varying positions (see Figure 3) along the length of the branch. Each section was faced using a plane and exposed to X-ray plates.

FIGURE 3

Relative position of slits, sections and injection points of the apple branch injected with radioactive phosphorus.

S ≡ Section.


S 14
S 13
8"
SLIT 3
S 13a
4"
10"
6"
8"
10"
8"
6"
10"
S20
SLIT 5
S19
S18
S17
S16
S15
S15a
SLIT 4
S12
6"
6"
5"
5"
5"
3"
3"
6"
4"
S11
S11a
S10
S9
SLIT 2
S9a
SLIT 1
S8
S7
S6
S5
S4
S3
S2
4" INJECTION 2
4" INJECTION 1
4"
S1

Detection of Living Material in Sections

For each transverse section cut a corresponding section was taken at the same position for the detection of living cells. Sections were placed in a 0.5% solution of 2, 3, 5 triphenyl-tetrazolium chloride (T.T.C.) and observations made for the formation of a red precipitate. The reduction of T.T.C. by cell dehydrogenases causes the formation of a red precipitate, enabling living cells to be detected (Fahn and Leshem, 1963).

Dye Injection

Basic fuchsin, at a concentration of 0.1%, was injected into a branch on a separate tree using the same method as adopted for the injection of P^{32}. Approximately 2.5 liters were allowed to enter the branch before it was removed and sectioned. Sections were taken every 4 inches along the branch until the presence of the dye was not observable.

Development of X-ray Plates

Kodak Liquid X-ray Developer Type 2 was used in the development of all plates and after passing through a stop bath of acetic acid, Kodak Liquid X-ray Fixer and Replenisher was used to fix the plate. All development was done

at a constant temperature of 68°F for 4½ minutes to ensure a qualitative comparison of intensity of radiation.

Results

The presence of P^{32} in the tree was indicated by radioautographs of the transverse sections and the slits cut through the branch. By far the highest concentration appeared in the outer part of the branch probably including the current phloem and xylem. Figures 4-13 show this result.

Some P^{32} was present in the wood of the branch. Figures 5-8, autoradiographs from Sections 1-8 (see Figure 3), show that movement of P^{32} occurred down (Figure 5, Sections 1 and 2) and upwards (Figures 6-8, Sections 3, 4, 5, 6, 7, and 8) from the point of injection. In Section 8 (Figure 8), there is an increased concentration of P^{32} in the wood. This is thought to be due to the presence of Slit 1 (Figure 3) which is approximately 5 inches further along the branch. The P^{32} possibly exuded from the cut bark of Slit 1 and migrated to the centre of the branch. This effect is seen in Section 11a (Figure 9), where there appears to have been movement from the bark, inwards along the cut surface. The concentration of P^{32} is greater in

FIGURE 4

Autoradiograph following exposure of X-ray plate in Slit 1 (see Figure 3) for 30 minutes.

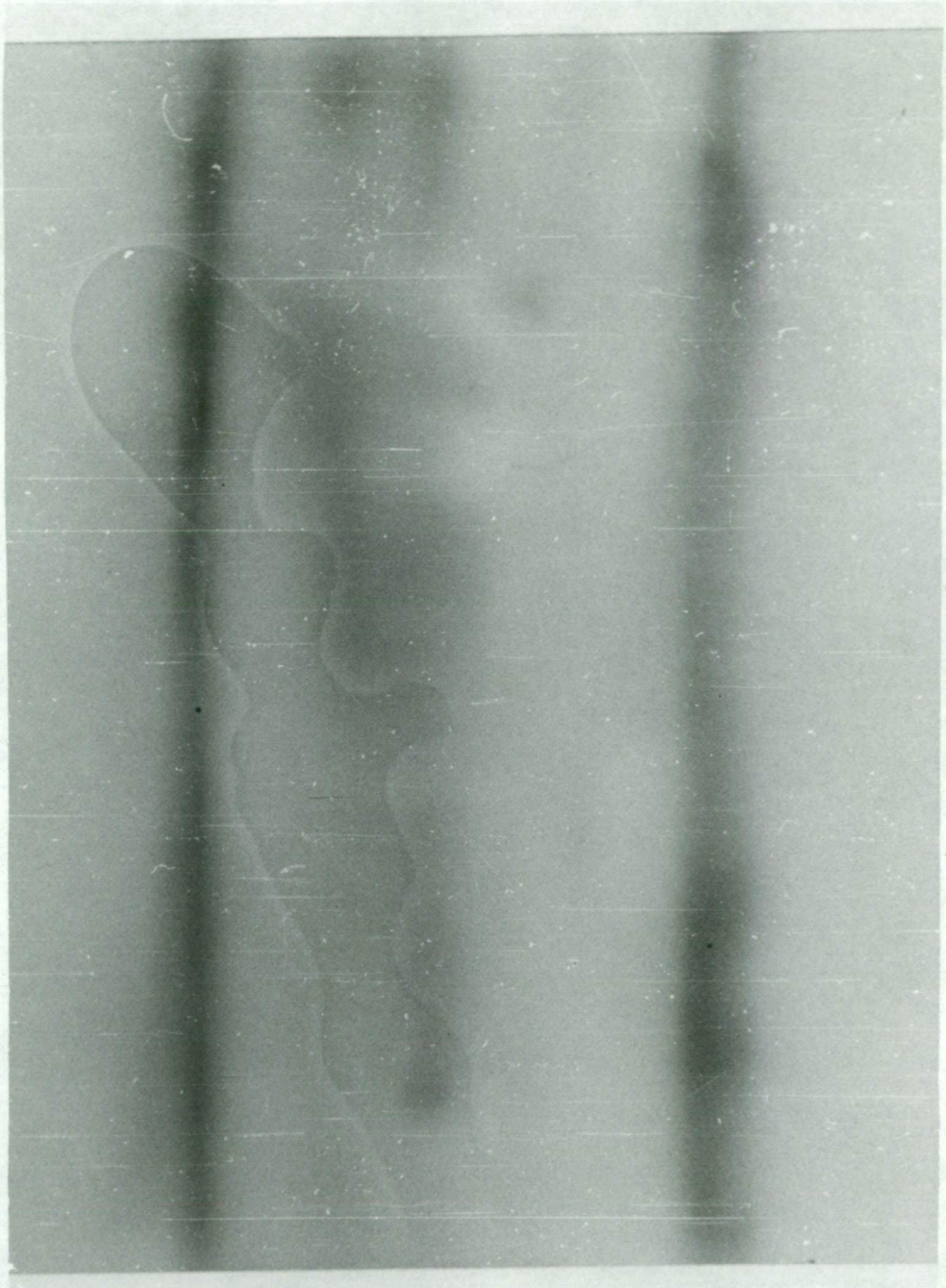


FIGURE 5

Autoradiograph of Sections 1 (above) and 2 (below) both taken from below the point of injection. Exposure time 4 hours.

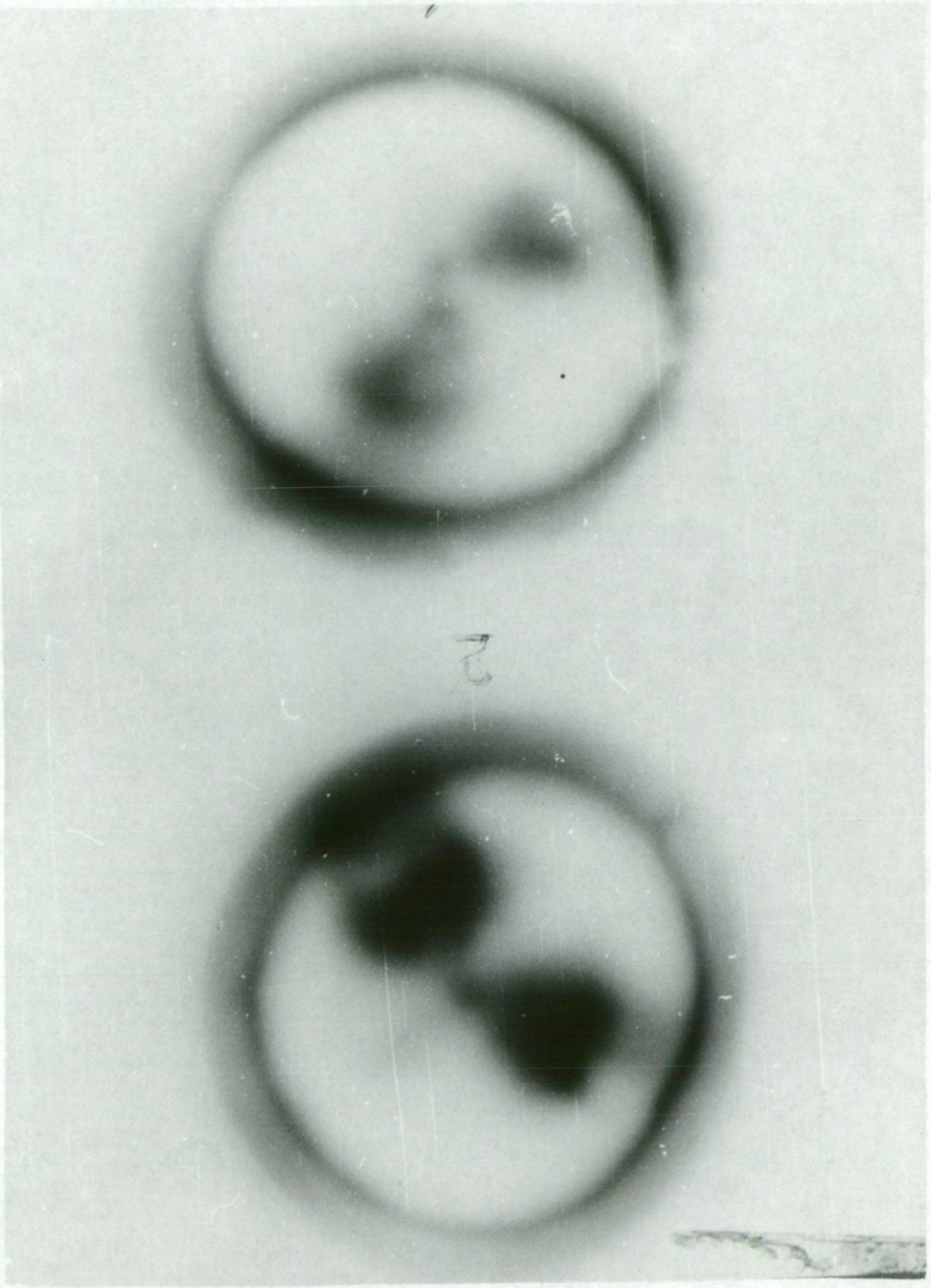


FIGURE 6

Autoradiograph of Section 3 (above) and 4 (below). A decrease in concentration of P^{32} can be seen from Section 3 to Section 4.

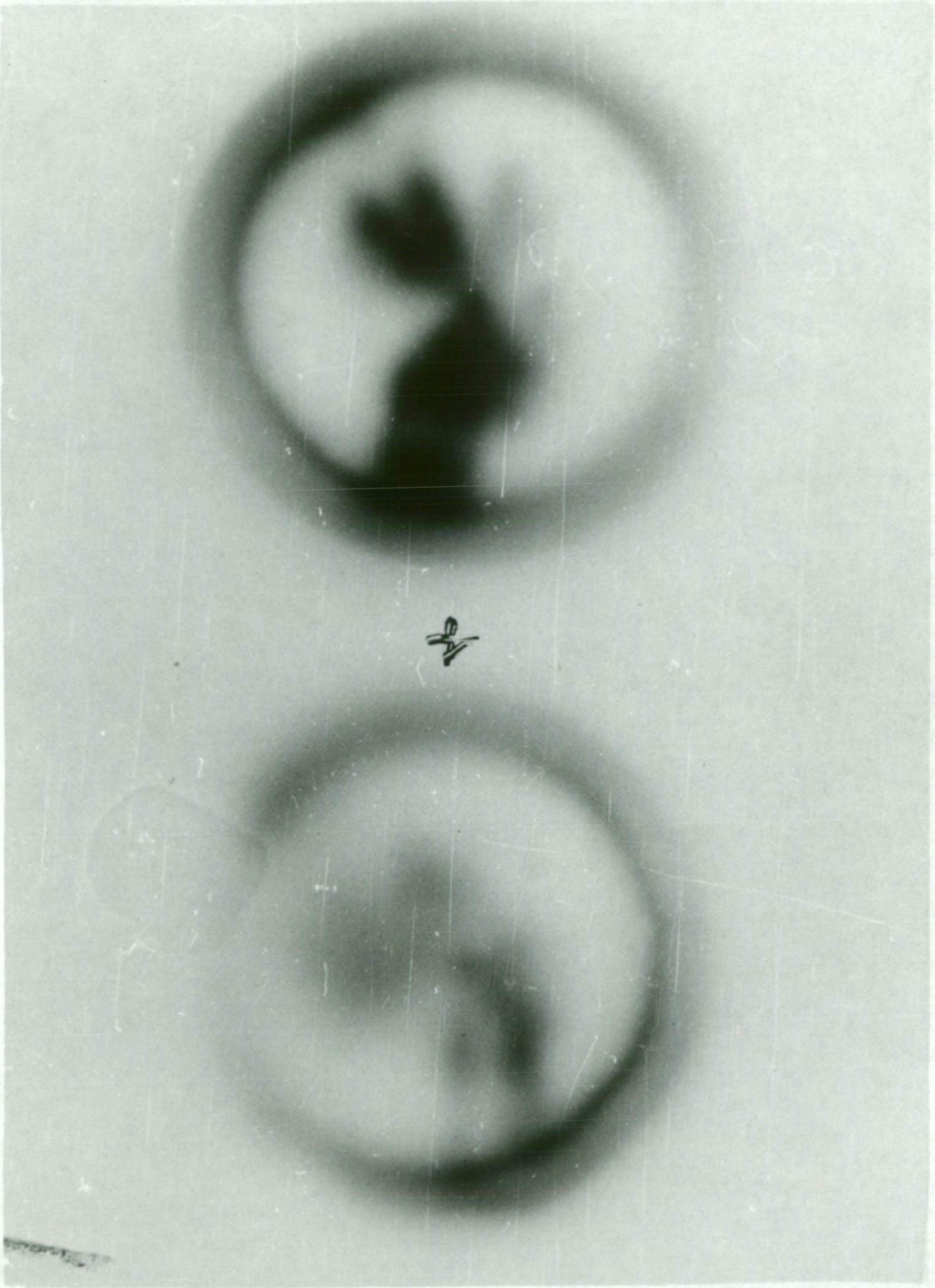


FIGURE 7

Autoradiograph of Section 5 (above) and 6 (below). An apparent concentration of P^{32} in the pith is clear.

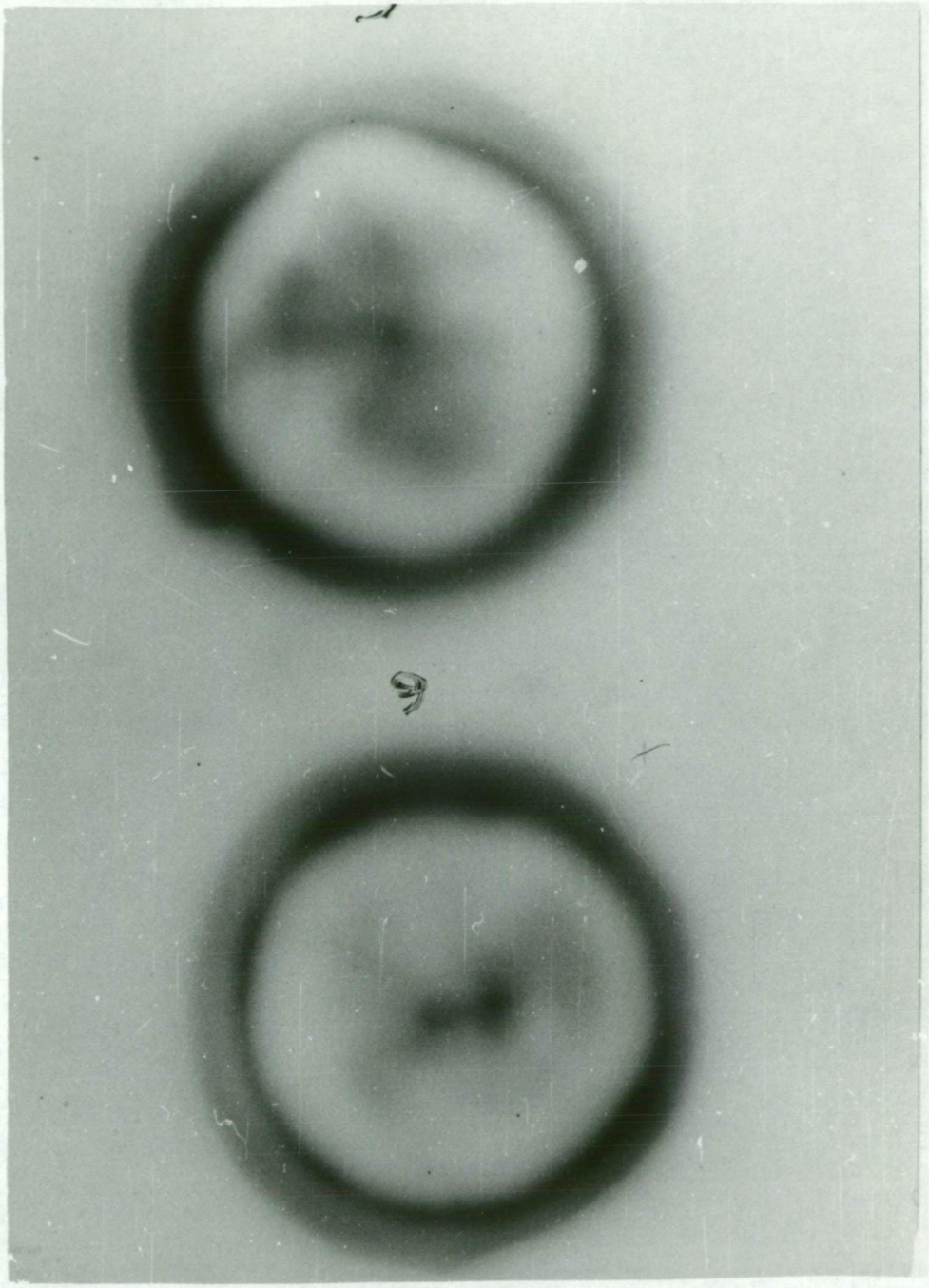


FIGURE 8

Autoradiograph of Section 7 (above) and 8 (below). The concentration of P^{32} has increased from Section 7 to Section 8 as Slit 1 is approached.

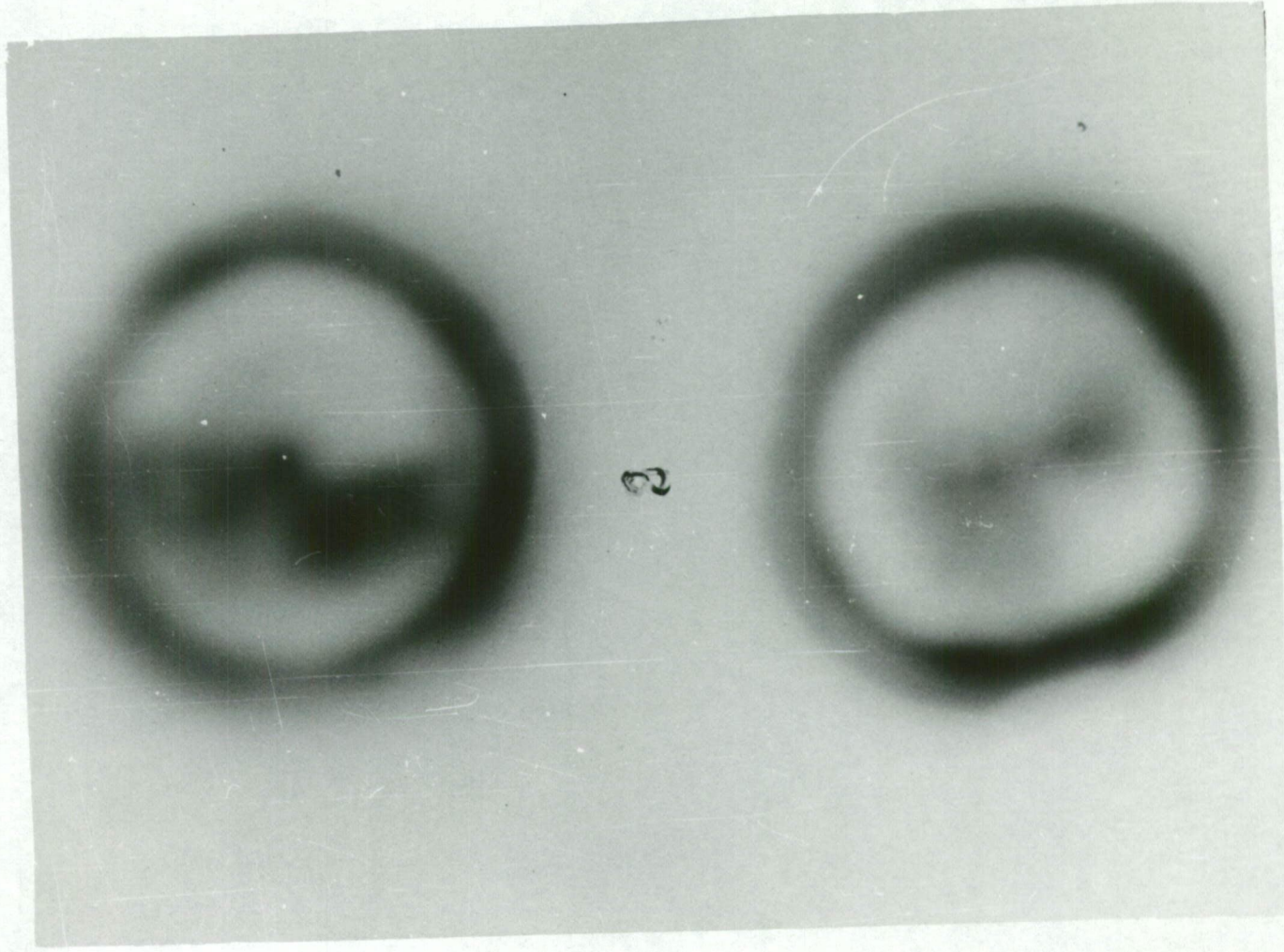


FIGURE 9

Autoradiographs of Sections 10 (above) and 11a (below). In Section 11a the effect of the slit can be seen. The P^{32} has migrated inward from the phloem and xylem and concentrated to some extent in the pith.

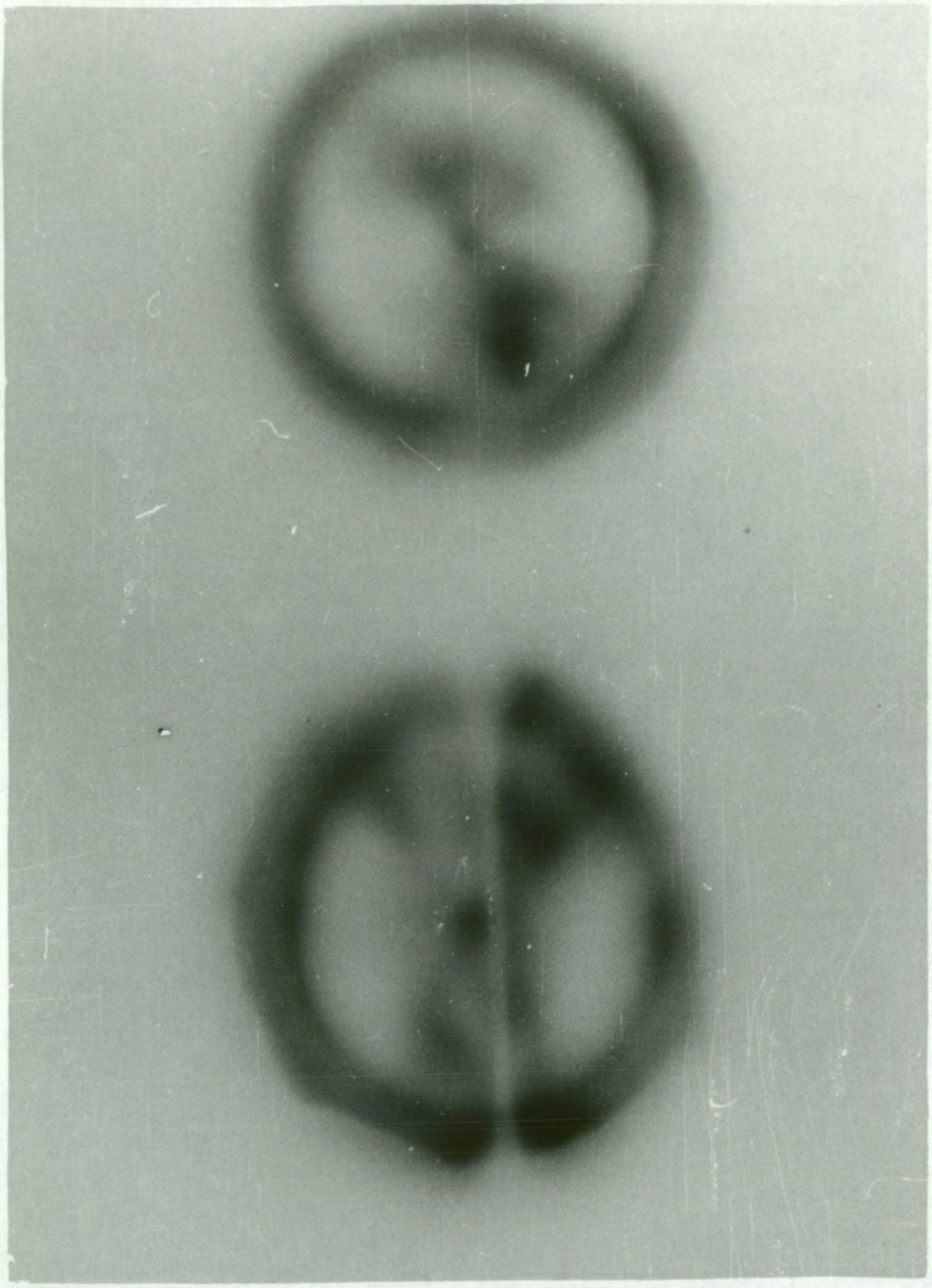


FIGURE 10

Autoradiograph taken from Slit 5. A slight concentration of P^{32} in the pith can be seen.

FIGURE 11

Autoradiographs of Section 17 (above), 16 (centre) and 15 (below). An increase in the concentration of P^{32} in the pith can be seen as the Sections approach Slit 5.

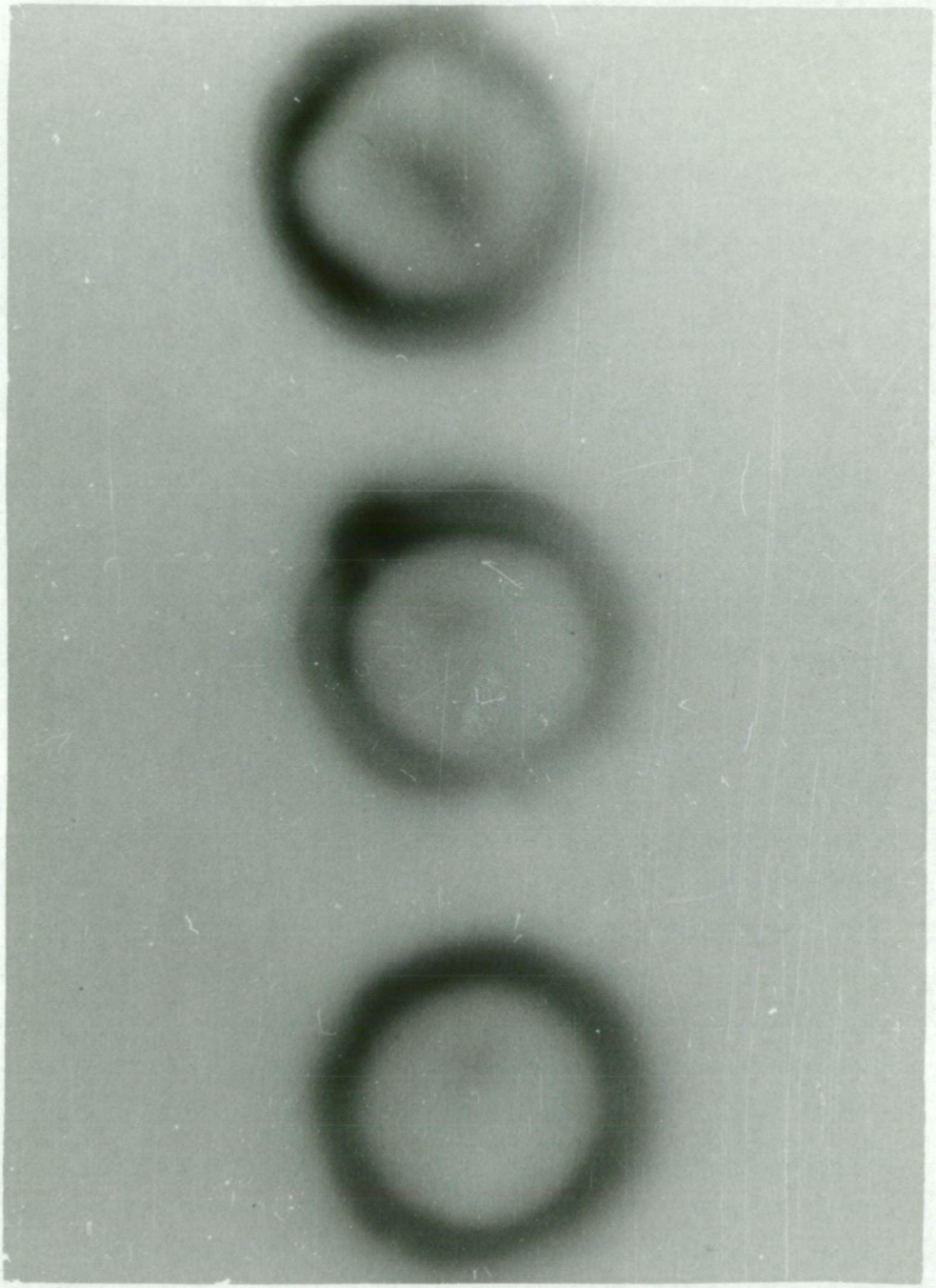


FIGURE 12

Autoradiograph of Section 18 (above) and 19 (below). The increased concentration of P^{32} in Section 19 (4" from slit 5) is obvious. P^{32} concentration in Section 18 is not as great as in Section 17 (Figure 11).

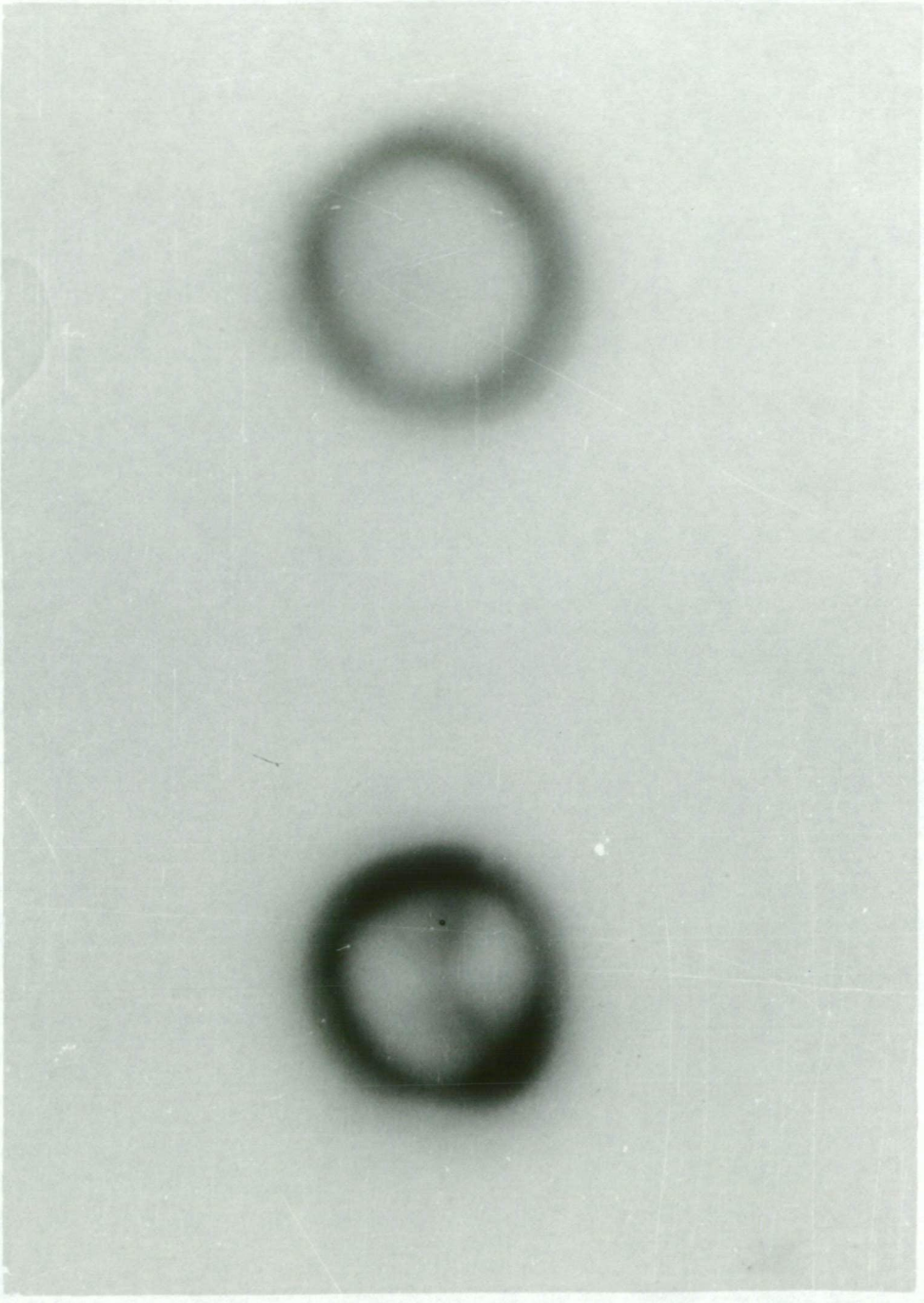


FIGURE 13

Autoradiograph taken from Slit 3, illustrating the P^{32} concentration in the pith of the branch.

the position of slit 2 (Figure 9, Section 11a) than six inches behind the slit (Figure 9, Section 10).

Figures 10 and 13 indicate the presence of a concentration of P^{32} in the pith of the branch. It is considered that this is a result of the methods used rather than being a real effect. Figures 11 and 12 show Sections 15-19 which are cut progressively away from Slit 4 and approaching Slit 5. A gradual decrease in the concentration of P^{32} in the wood and pith can be seen in Sections 15-18 followed by an increase in Section 19 as the 5th slit is approached.

With regard to P^{32} accumulation in the pith, possibly this tissue acted more effectively as a conductive path for P^{32} accumulated in the area of the slits, than the wood of the branch.

Sections treated with T.T.C., including Sections 9-18, displayed a red precipitate in the outer part of the branch and the pith. This indicated that the pith to at least Section 9 was living. The age of the branch in this area was estimated to be 34 years. This result could not be repeated in the branch of another tree. Despite this and the fact that the presence of radioactivity may have

provided sufficient potential for the reduction of T.T.C., it is quite possible for this section of the branch to have contained living cells. Ziegler (1964) mentioned that quite frequently the cells of the pith may remain alive for a long time and are capable of storage of reserve materials. If the cells of the pith of the injected branch were in fact alive this would have to some extent resulted in an accumulation of P^{32} in these tissues.

When basic Fuchsin was injected into the branch of another tree the dye was observed to travel only in the wood, directly above and below the injection holes. The dye persisted along the length of the branch for 3'6".

Discussion

Apart from the effect of the injection method and the presence of the five slits cut, there appeared to be no real movement of phosphorus in the wood of the branch. That this was an effect of the injection method was supported by the results of the dye injection experiment. It is difficult to explain the absence of dye from the current xylem and phloem but the size of the basic fuchsin molecule may have restricted its movement in these tissues. The limited appearance of dye in the branch was most probably

due to,

(a) an absence of dye in the translocation stream,
(b) slits were not cut in the branch into which the dye was injected.

The possible detection of living cells in the older wood of the tree may have been anticipated as the apple tree is capable of accumulating large quantities of carbohydrates. Cells of a parenchymatous nature are capable of acting as storage cells and remaining alive so long as "cells remain in contact with one another and the assimilate translocation system" (Ziegler, 1964).

Although this work was terminated following the failure of the field survey (Chapter 1, Section III) to establish any relationship between the phosphorus status and incidence of dieback in trees, the results are of interest. Despite some evidence to the contrary, the possibility of movement of phosphorus in the pith, providing cells in this tissue are living and have a living connection with the external phloem and xylem, is not completely absent.

CHAPTER 2

The Influence of Sugars on the Resistance of Apple Wood to Decay by *Trametes versicolor*.

Introduction

Mineral nutrition has been associated with the incidence of Polystictus dieback in Australian apple trees (see General Introduction). However the results of a field survey of Southern Tasmanian apple orchards failed to provide evidence to associate the phosphorus, potassium or calcium levels in the wood of current seasons growth of trees with the occurrence of dieback (see Chapter 1).

Appreciating the distinction between the two aspects of tree nutrition, current mineral nutrition and reserve compounds within the tree acting as a nutrient source, an hypothesis was postulated with reference to tree nutrition, to describe the sometime parasitic nature of T. versicolor.

It was postulated that reserve nutrient levels in the wood of apple trees may play an important part in the development of wood decay by T. versicolor. In the case of high reserve nutrient levels, the fungus may use these as substrates and not destroy the wood structure, whereas when reserve nutrient levels are low the fungus has virtually only the structural elements of the branch on which to survive and proliferate. Hence in the former case an epiphytic situation may exist, while in the latter the

fungus has become parasitic.

Severe phosphorus deficiency in sand culture trees resulted in an increased susceptibility of limbs to attack by T. versicolor (Wade, unpubl.). A variety of effects resulting in the inability of the trees to accumulate reserves could be expected from a phosphorus deficiency. Mochizuki (1962) concluded that fruiting generally caused a decline in tree vigour, this being due to a reduction in the function of the small roots because of inadequate carbohydrate supplies. Overcropping would then reduce the ability of trees to accumulate reserves effectively. Other cultural factors that may be expected to result in a depletion of, or inability to accumulate reserves, include water logging and drought conditions, root damage, heavy pruning and mineral element deficiencies.

The present section of this thesis contains a review of literature concerning the carbohydrate nutrition of apple trees and literature related to the role of T. versicolor in the breakdown of wood. This is followed by the results from in vitro methods used to determine the influence of the carbohydrate and nitrogen supply on the susceptibility of apple wood to decay by T. versicolor. In addition the influence of the nutrient supply on the

ability of T. versicolor to produce polyphenol oxidase (enzymes partly responsible for wood breakdown) is recorded.

II A - LITERATURE REVIEW

Factors Influencing the Distribution and Supply of Carbohydrates in the Apple Tree.

Introduction

Carbohydrates are basic to the needs of any tree as they are essential for the formation of structural elements and other organic constituents. Their variety and importance of function is illustrated in the following quotation from Kramer and Kozlowski (1960). "They are the chief constituents of cell walls; they form the starting point for the synthesis of fats and proteins; large amounts are oxidised in respiration; and whatever is left accumulates as reserve food. Soluble carbohydrates increase the osmotic pressure of the cell sap, and such carbohydrates as the pentosans, pectic compounds, gums and mucilages increase the water holding capacity."

Fruiting trees such as the apple, have additional demands on carbohydrate supplies as large amounts of these compounds are required for fruit formation. This has immediate commercial implications and stresses the need for an adequate knowledge of factors affecting the supply and movement of carbohydrates in trees.

Kramer and Kozlowski (1960) have estimated that approximately 75% dry weight of apple trees is composed of carbohydrates. In further emphasizing their importance

it is interesting to see the estimated annual usage of carbohydrates in a mature tree. Murneek (1942) quotes Heinicke in the following figures.

Estimated Carbohydrate Requirement in Pounds, of an Apple Tree Producing 25 Bushels of Fruit per Year.

	Carbohydrates (lbs.)
Fruit Crop	192
Leaf Production	62
Structural Tissues	187
TOTAL	441 lbs.
Respiration	100 - 125 lbs.

Two forms of carbohydrates within a tree are generally recognised, these being "active" and "reserve" or "storage". The distinction as Priestley (1962) points out is one of convenience but it is necessary for a full appreciation of carbohydrate formation, movement and utilization. "Active" are those currently involved in tree function while "reserve" carbohydrates refer tothose produced in excess of immediate requirements and stored in perennating organs to be subsequently used in support of new growth. It is appreciated that a dynamic relation exists between the two types.

Seasonal Effects on Distribution of Carbohydrates Within the Tree

As with mineral elements, the fluctuations in levels of carbohydrates in apple trees should be viewed in the light of the different growth phases of the tree. Thus initial depletions occur at bud break when reserve materials contribute all requirements. This is generally followed by a replacement as the leaves of the new growth contribute photosynthates in support of further development. Rapid extension growth and demand from fruiting then causes further depletions. After meristematic activity declines there is a further build up as photosynthates exceed the immediate requirement of the plant.

A number of factors to some extent complicate this pattern. All tissues, including the old xylem and pith, must be taken into consideration. Different carbohydrate fractions within the tree tend to display different seasonal fluctuations in the various tissues. The demands of fruiting affect the ultimate distribution of photosynthates. The interconversion of one carbohydrate fraction to another (e.g. starch-soluble sugar) takes place.

Swarbrick (1927) quotes Mer and Russow in making observations on the seasonal starch change in apple

branches. Swarbrick mentions that the seasonal change in starch content shows on the whole two maxima and two minima, "the maxima occurring respectively at leaf fall and very early in the Spring; the minima at the time of maximum leaf growth and early Winter". It is understood that maximum leaf growth refers to development before shoot extension. The Winter minimum has been generally assumed to be a result of low temperatures but has recently been explained more explicitly in terms of starch-sucrose interconversions (Siminovitch et al., 1953, Priestley, 1960).

Mochizuki and Hanada (1957) looked closely at the seasonal changes of starch in young apple trees. Their work elaborated earlier investigations. It was demonstrated that starch content "decreased at the beginning of shoot growth and then increased slightly at the time of temporary retardation of shoot growth". At the time of most active shoot growth "starch decreases again and begins to increase at the end of shoot growth, and reaches its maximum content at leaf fall". These changes were more obvious in the roots than in the aerial parts of the tree. With regard to content "reserved starch is partially removed to the wood tissues of the aerial parts of the tree previous to the buds swelling". Wood containing starch was

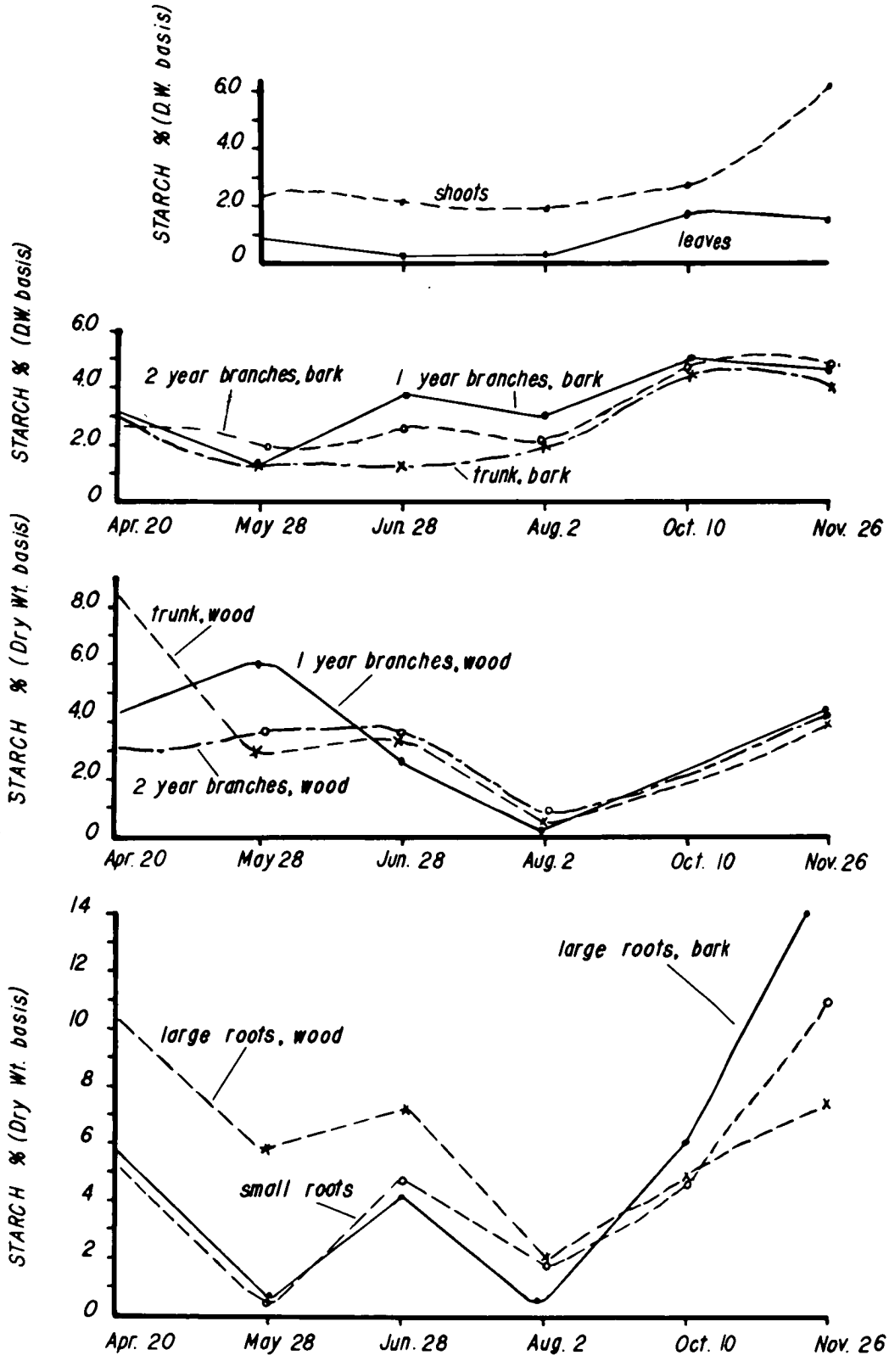
shown to act as a "sink" supplying the bark, consequently levels in the wood were higher than those in the bark at the beginning of growth. There was a general decrease in levels recorded in both the wood and the bark until cessation of meristematic activity. At this time bark levels first rose, then following movement to the wood, concentrations in both tissues were equalised. Figure 14 taken from Mochizuki and Hanada (1957) demonstrates these changes.

In addition to starch it is convenient to recognise three other carbohydrate fractions, these being glucosides (phloridzin), soluble sugars and hemicellulose (Priestley, 1962). Priestley (1960) demonstrated that phoridzin did not follow closely the variation of other carbohydrate components in relation to phases of growth. Absence of explanation of this behaviour, which is at variance to "normal changes", could possibly be due to the lack of knowledge (Pridham, 1960) of the function of the group of compounds in question.

Seasonal changes of soluble sugars display similar trends to starch. Differences in the soluble sugars and starch curves can generally be attributed to the starch-soluble sugar interconversion. Priestley (1960) mentioned

FIGURE 14

Seasonal changes in the starch content of apple trees (after Mochizuki and Hanada, 1957).


STARCH % (D.W. basis)
6.0
4.0
2.0
0
shoots
leaves
STARCH % (D.W. basis)
6.0
4.0
2.0
0
2 year branches, bark
1 year branches, bark
trunk, bark
Apr. 20
May 28
Jun. 28
Aug. 2
Oct. 10
Nov. 26
STARCH % (Dry Wt. basis)
8.0
6.0
4.0
2.0
0
trunk, wood
1 year branches, wood
2 year branches, wood
Apr. 20
May 28
Jun. 28
Aug. 2
Oct. 10
Nov. 26
STARCH % (Dry Wt. basis)
14
12
10
8
6
4
2
0
large roots, bark
large roots, wood
small roots
Apr. 20
May 28
Jun. 28
Aug. 2
Oct. 10
Nov. 26

that "at the beginning of the year before growth commenced, the amounts of starch were observed to increase while amounts of sugar decreased, and conversely at the close of the year when the trees became "dormant", the amount of soluble sugar increased while starch decreased".

Initially the role of hemicellulose within the plant was not well defined. It is now considered to be an important reserve material. Murneek (1928) has shown that new growth of fruiting spurs is constituted of 17-22% hemicellulose compared with 1-4% of starch. Afrikjan et al. (1954) studying vine varieties concluded that hemicellulose was an important reserve carbohydrate and can take an active part in carbohydrate metabolism. Priestley (1960) demonstrated a marked seasonal fluctuation of hemicellulose and also stressed its importance as a reserve material.

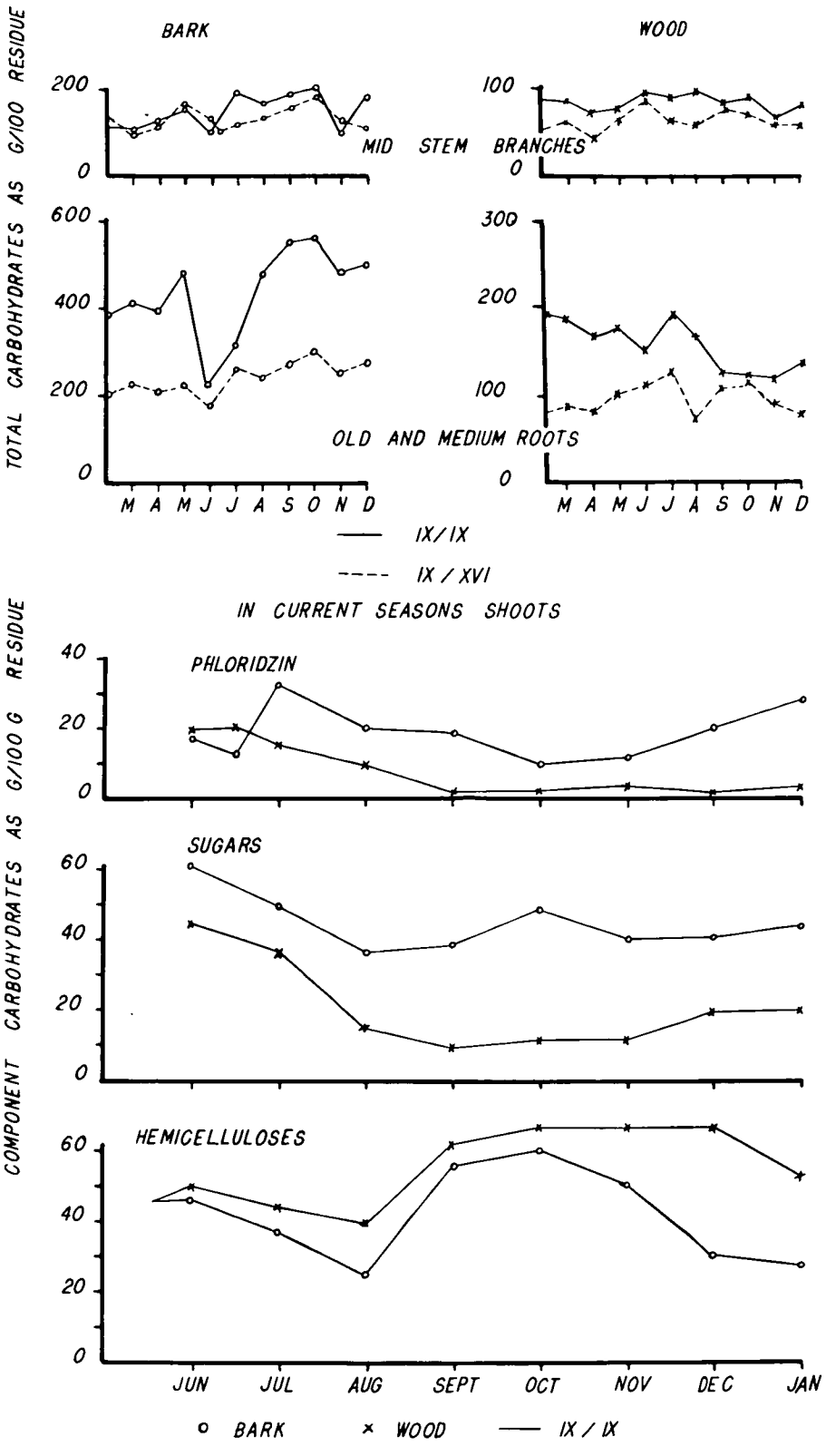
Figure 15 published by Priestley (1960) illustrates the changes in levels of phloridzin, sugars, hemicellulose and total carbohydrate.

Fruiting and Carbohydrate Distribution

Fruiting effects on carbohydrate distribution were investigated in detail by Mochizuki (1962). Trees grown

FIGURE 15

Seasonal changes in various carbohydrate fractions within the apple tree. (after Priestley, 1960).


BARK
WOOD
TOTAL CARBOHYDRATES AS G/100 RESIDUE
200
0
100
0
MID STEM BRANCHES
600
400
200
0
300
200
100
0
OLD AND MEDIUM ROOTS
M A M J J A S O N D
IX/IX
IX / XVI
IN CURRENT SEASONS SHOOTS
COMPONENT CARBOHYDRATES AS G/100 G RESIDUE
PHLORIDZIN
40
20
0
SUGARS
60
40
20
0
HEMICELLULOSES
60
40
20
0
JUN
JUL
AUG
SEPT
OCT
NOV
DEC
JAN
BARK
WOOD
IX / IX

in solution culture, divided into bearing and deflorated plots, were treated with radioactive carbon dioxide at different times throughout the growing season and subsequent movement of the labelled carbon traced. Treatments were applied on June 3rd, July 7th, July 30th and August 25th, which corresponded to 26 days, 60 days, 83 days and 109 days respectively after flowering. Mochizuki summarizes his results as follows:

"1. Most of the carbon assimilated on June 3rd was distributed to the leaves and the rest was divided among the other parts, among which the shoots and the small roots were supplied with a relatively large amount of the carbon. This trend of carbon distribution was almost equally recognized in each plot, for little carbon was distributed to the fruits at this time of the year.

2. Of the carbon assimilated on July 7th a considerable difference in quantity was found in the small roots, the roots of bearing trees showing virtually no change from the previous treatment while a considerable increase was found in the roots of the deflorated plot.

3. On July 30th less carbon was distributed to the roots of the deflorated trees compared with the case of the previous treatment but distribution to the trunk

increased. In the bearing trees the distribution ratio to the leaves and fruit increased and the ratio to the small roots and trunk decreased in contrast to the previous treatment.

4. The distribution ratio to the trunk of the deflorated trees on August 25th. had decreased and the ratio to the large roots increased in compensation. In the bearing plant such an increase in the large roots was not seen and carbon supplied mainly roots, leaves and shoots."

It was concluded from the above observations that fruiting was generally related to a decline in tree vigour, this being a result of a reduction in the function of the small roots because of inadequate carbohydrate supplies. (Eaton and Joham (1944) using cotton plants demonstrated that the presence of available carbohydrate was necessary for the uptake of mineral elements.) In addition reserves in the trunk and root system were at a lower level which would probably be detrimental to early growth in the following season.

Ursulenko (1962) also commented on the fact that in years of high yield, the "level and nature of metabolic activity of the root system of the apple tree is frequently

disrupted because of the insufficient afflux of the products of photosynthesis from the leaves to the roots". This as has been mentioned, causes a reduction in root function during the time of active vegetative growth.

Nitrogen and its Effect

Ritter (1958) reported that there were no consistent effects of nitrogen fertilization on amounts of total sugars, alcohol insoluble materials and total carbohydrate in the various tissues. This was reported earlier by Hopkins and Greve (1931) when it was found that there was little or no correlation between nitrate treatment and percentage of soluble sugars in apple fruit. Beattie (1948) applied three levels of sulphate of ammonia (high $7\frac{1}{2}$ lbs., medium 5 lbs., low $2\frac{1}{2}$ lbs.) to trees over a period of four years. In terms of percentage dry weight the high nitrogen treatments had a lower level of starch than the low nitrogen treatments but in absolute amounts the situation was reversed due to the larger amount of growth in the former treatment. There was a difference of approximately 30 mgms. of starch in the twigs of the two treatments in September when the maximum variation is seen. Absolute amounts of sugar displayed a variation of approximately 20 mgms. between high and low nitrogen treatments.

Unfortunately, in this work only shoots and twigs were sampled giving a rather incomplete picture of the carbohydrate status as affected by different nitrogen treatments.

Benedict and Brown (1944) working with two grasses, Agropyron smithii and Bouteloua gracilis, showed that lack of nitrogen reduced total dry weight of the plants but favoured the growth of the roots as compared to that of the tops. Carbohydrate analysis indicated that the effect of lack of nitrogen was to increase the percentage of sucrose and starch while addition of nitrogen to previously deficient plants decreased these quantities. Jones et al. (1965) demonstrated that in Spring and Autumn under a system of monthly cutting, nitrate of soda, sulphate of ammonia and urea depressed soluble carbohydrates in perennial ryegrass, cocksfoot and timothy.

The source of nitrogen is also an important factor to be considered. Priestley (1960) quotes Sideris in making a distinction between nitrate and ammonium nitrogen when supplied to the pineapple. Ammonium salts were absorbed more readily, and with greater depletion of carbohydrate reserve, than were nitrates.

It appears that short term effects of nitrogen application are not reflected to any extent in the overall carbohydrate status in the apple tree. Little is known of the result on the tree of long term nitrogen applications (and when applied in different forms), particularly on root growth and accumulation of carbohydrate reserves.

The deficiency or excess of elements other than nitrogen on the distribution and supply of carbohydrates in the apple tree has not been studied in any detail. It can only be surmised from already known effects of the various elements on growth what the resulting changes in carbohydrate levels will be. A complex and commercially significant interplay of the various elements is to be expected. Nightingale (1942b) demonstrated such an effect in the pineapple. "When potassium was low and the absorption of it in consequence limited, carbohydrate accumulation was also curtailed. In turn, although no phosphate was applied, the low level of nitrate permitted fairly free absorption of phosphate."

Carbohydrate Accumulation as Influenced by Water Supply

Generally water stress is reflected in a reduction of stomatal activity. Magness et al. (1932) found that

the stomata in the foliage of non-irrigated apple trees remained open only half as long as those of trees on irrigated plots. They demonstrated that differences in sugar and starch content of irrigated and dry leaves were small, but consistent throughout the day. However the starch content of the wood and bark of irrigated trees was markedly higher than that of the corresponding dry trees. It was suggested that under dry conditions the starch-sugar ratio in the storage organs of the tree is modified so that a large proportion of the carbohydrates is in the form of sugars. This indicates a similar effect on carbohydrates as cold weather conditions.

McCune (1958) determined the movement of carbohydrate by using radioactive phosphorus which was found to move only in the presence of carbohydrates. Under conditions of increasing moisture tension McCune found that movement of carbohydrate from the leaves of sunflower and soya bean increased. There was a corresponding decrease in carbohydrate production indicating impaired functioning of stomata. It was concluded that an increase in water stress speeds up carbohydrate movement from mature leaves and decreases carbohydrate production and plant elongation.

A variety of factors influence the carbohydrate regime of apple trees. Virtually all are reflected in tree "vigour" and ability to fruit. An adequate knowledge of these factors is required to be able to take practical measures to ensure a balance between maximum "tree health" and maximum crop production.

II B - LITERATURE REVIEW

Trametes versicolor and its Role in Lignin Breakdown.

Lignin is one of the basic structural components of wood. Henderson (1963) summarises the structure of lignin as follows. "It consists only of carbon, hydrogen and oxygen, the carbon content being higher than it is in carbohydrates, and the presence of certain groups, such as methoxyl and hydroxyl, has been demonstrated. An aromatic structure is indicated by the release of vanillin on the oxidation of softwood lignins by nitrobenzene under alkaline conditions. Hardwood lignins yield, in addition to these, p-hydroxybenzaldehyde. There is also evidence of a side chain of three carbon atoms being attached to some of the aromatic rings. The generally accepted theory, based on this information, is that lignin is built up of phenyl-propane units, having methoxyl and hydroxyl groups attached to them. The nature of the attachments between the units is not yet known. They may, according to Freudenberg, condense and form benzofuran and benzopyran rings which are linked in chains, or they may be linked in chains with carbon-carbon and carbon-oxygen linkages."

Lignin is particularly resistant to attack by microorganisms. Wood destroying fungi, perhaps the principle agents responsible for the destruction of lignin, have been divided into white and brown rot fungi.

Fahraeus et al. (1949) quotes Falk as finding in 1926 that brown rot fungi attack cellulose but not lignin whereas the white rot fungi primarily attack lignin, but later cellulose. This resulted in the terms corrosive rot and destructive rot for white and brown rot fungi respectively.

Trametes versicolor, a white rot fungus, has been used widely in investigations into the enzyme production of this group of fungi. Three groups of enzymes have been shown to be produced by T. versicolor. Fahraeus (1950) mentions that, like other lignin decomposing fungi, T. versicolor forms large amounts of phenoloxidase (laccase and tyrosinase) and has shown (Fahraeus et al., 1949) that phenols and aromatic amines are readily oxidised indicating that the active enzyme is laccase. Extra-cellular oxidase production has often been used as a method of differentiation between white and brown rotting fungi (Davidson et al., 1938) and as a taxonomic tool (Nobles, 1958). Henderson (1963) and Farmer et al. (1959) demonstrated the presence of an extra-cellular aromatic alcohol dehydrogenase which was shown to dehydrogenate a number of primary aromatic alcohols.

In addition it is known that T. versicolor produces at least four cellulolytic components (Pettersson

et al., 1963). Some evidence also exists (Blumer and Nuesch, 1962) to suggest the production of a pectinase enzyme.

The precise role of the extra-cellular enzymes of wood rotting fungi is not well defined. This is possibly due to the fact that the complex structure of lignin has not yet been described. Henderson (1955) demonstrated the release of aromatic compounds (vanillic acid and syringic acid) from wood rotted with T. versicolor. It was concluded that these compounds were probably released by the action of extra-cellular enzymes produced by the fungus and that such molecules were then rendered available to microflora under natural conditions. Later it was shown (Henderson, 1963) that T. versicolor did not grow on p-hydroxybenzaldehyde, ferulic, syringaldehyde or vanillin but the three latter compounds were metabolized through synringic and vanillic acids.

When glucose was added to a basal mineral salts medium, in which the fungus was growing, the amount of conversion of aromatic materials was greater, "probably due to a combination of phenoloxidase activity and of decomposition". (Henderson, 1963). Law (1959) considers the association of laccase with lignin decomposition is in the

oxidation of phenolic substances. Law concluded that the bulk of the lignin was little affected by this enzyme. Gadd (1957) attributes the removal of toxic monomeric aromatic substances to the action of laccase.

III - EXPERIMENTAL

Methods

Two methods were adopted to investigate the hypothesis mentioned in the Introduction.

(a) "Wood Block" - where the fungus was grown on apple wood in culture vessels containing different basic nutrients incorporated in an agar medium. Details of the treatments and media used in these experiments are described in Section D of this method.

(b) Parallel experiments where the fungus was grown on apple wood in solution culture, having the same nutrient composition as some of the wood block experiments, to determine their effects on the production of extracellular polyphenol oxidase by the fungus.

A. Wood Block

A modification of the soil block technique as used by Aplin and Da Costa (1954) was developed. T. versicolor was grown in the presence of blocks of apple wood in culture vessels consisting of glass jars, 7 cms. diameter and 13 cms. high, with metal screw top lids. Each vessel contained 200 mls. of defined media. Blocks of apple wood 5 x 2.5 x 0.6 cms. (cut with the longitudinal axis of the block corresponding to the longitudinal section of the tree)

were dried at 80°C for three days before being individually weighed and numbered. In a dried condition blocks were then placed in empty jars and sterilized by autoclaving for one hour. The media was sterilized separately by autoclaving for 20 minutes. After aseptically placing a single block of wood on the surface of the agar in each jar, vessels were inoculated with two small mycelial pads of T. versicolor approximately 0.5 cm. square, placed on either side of the block. Following inoculation the culture vessels were incubated at 28°C and five replicates from each treatment harvested at monthly intervals for six months.

The percent dry weight loss of blocks due to decay by T. versicolor was estimated after separating the fungus from the wood block, drying at 80°C for three days and calculating the difference between the original and decayed weight.

The total dry weight of mycelium produced in each culture vessel was also estimated. This included mycelium from both the surface of the block and surface of the media. As with the wood blocks the mycelium was dried at 80°C before weighing.

Experiment 1

Four threatments were set up, these being (a) a complete nutrient treatment, the media containing both carbon and nitrogen sources (referred to as Complete).

(b) the same as (a) but omitting the carbon source (Complete - C).

(c) the same as (a) but omitting the nitrogen source (Complete - N).

(d) both the carbon and nitrogen sources being omitted from the media (Complete - C - N).

Experiment 2

The second experiment consisted of repeating the first but using inorganic nitrogen rather than Bacto-Peptone as a nitrogen source. Yeast was added to the media to enhance the early establishment of the fungus and the glucose level was raised to 3.0%.

Experiment 3

The third experiment was conducted with six treatments each with the same nitrogen level but having six different carbon levels.

Experiment 4

Finally to determine the relationship between soluble materials in the wood and its ability to withstand fungal attack, wood blocks were (a) extracted for 24 hours with 75% aqueous methanol to remove soluble sugars and nitrogen and (b) extracted for 24 hours with 75% aqueous methanol, 8 hours with acidified methanol followed by 8 hours with 96% methanol (to solubilise starches), then a further 24 hours with water to remove starches. In one treatment blocks were subjected to the above extractions then hydrolized with NH_2SO_4 to remove hemicelluloses.

The extracted blocks were then subjected to attack by the fungus grown on media containing a constant nitrogen level but different levels of carbon (see Section D of this method for details of treatments and media).

Thus in the above experiments a "model" system was established - the media acting partly as a nutrient source for the fungus where its effect on the wood could be studied under different nutrient conditions.

B. Solution Cultures for the Estimation of Extracellular Oxidase

T. versicolor was grown on wood in solution culture

to determine the extracellular polyphenol oxidase production. Cultures were grown in 100 ml. Erlenmeyer flasks each containing 30 mls. of media. Three wood blocks, 5 x 1.25 x 0.6 cms., were added to each flask before inoculating with 0.2 mls. of a mycelial suspension of T. versicolor. Mycelial and extracellular polyphenol oxidase production were estimated after incubation of the flasks for six days at 28°C.

Experiment 1A

Four treatments were established these being the same as in experiment 1, Section A of this Method. The effects of the different nutrient treatments on the production of polyphenol oxidases could be established.

Experiment 3A

Six treatments containing different levels of glucose as in experiment 3 were set up. The effects of different levels of glucose on the ability of the fungus to produce polyphenol oxidases could then be demonstrated.

Section D of this method contains details of the media used in these experiments.

C. Oxidase Determinations

The Warburg method proposed by Fahraeus and

Tullander (1956) was employed for the estimation of production of extracellular polyphenol oxidase. The reaction mixture consisted of:

20% KOH	0.2 mls.
Buffer pH 6.0	1.0 mls.
Substrate (0.1M Catechol)	0.4 mls.
Culture filtrate	1.0 mls.

D. Treatments and Media

Three basic media used in the experiments described in Section A and B of this Method are set out in Table 2 below.

Table 2 - Media

Nutrient	Media 1	Media 2	Media 3
Glucose	20.0 gms.	30.0 gms.	-
Sucrose	-	-	30.0 gms.
Bacto-Peptone	5.0 gms.	-	-
$(NH_4)_2HPO_4$	-	5.0 gms.	5.0 gms.
KH_2PO_4	1.0 gms.	1.0 gms.	1.0 gms.
$MgSO_4$	0.5 gms.	0.5 gms.	0.5 gms.
Agar	10.0 gms.	10.0 gms.	10.0 gms.
Yeast	-	10 mgms.	10 mgms.
Distilled Water	1000 mls.	1000 mls.	1000 mls.

Details of the media used for treatments in experiments 1, 1A, 2, 3, and 3A are set out in Table 3. Indication is given also of the presence or absence of nitrogen and the level of glucose in the media.

Table 4 gives details of media and treatments of experiment 4, Various extractions to which blocks were subjected in the different treatments are included. Media 3 in which the nitrogen levels are constant at 0.5% $(NH_4)_2HPO_4$, was used in all nine treatments with the level and type of carbon source being indicated.

Table 3

Media used for treatments of experiments 1, 1A, 2, 3 and 3A. The presence or absence of nitrogen and level of glucose in the media is also indicated.

	Treatment	Media 1	Media 2	Nitrogen	% Glucose
Experiment 1	Complete	+	-	+	2.0
	Complete - C	+	-	+	0.0
	Complete - N	+	-	-	2.0
	Complete - C - N	+	-	-	0.0
Experiment 2	Complete	-	+	+	3.0
	Complete - C	-	+	+	0.0
	Complete - N	-	+	-	3.0
	Complete - C - N	-	+	-	0.0
Experiment 3	Complete C 3.0%	-	+	+	3.0
	Complete C 2.0%	-	+	+	2.0
	Complete C 1.5%	-	+	+	1.5
	Complete C 1.0%	-	+	+	1.0
	Complete C 0.5%	-	+	+	0.5
	Complete C 0.0%	-	+	+	0.0
Experiment 1A	Same treatments, media and nitrogen and glucose levels as exper. 1 but agar omitted.				
Experiment 3A	Same treatments, media, nitrogen and glucose levels as exper. 3 but agar omitted.				

TABLE 4

Experiment 4

(Media 3 used in all treatments)

Treatment	% Sucrose	M added at 0.5% glucose equiv. (a)	W added at 0.5% glucose equiv. (b)	Wood blocks untreated	Wood blocks extracted with methanol for 24 hours	Wood blocks extracted with methanol then water for 24 hours	Wood blocks extracted with methanol and water then hydrolysed with NH_2SO_4
Complete C	2.0% 2.0	-	-	+	-	-	-
" C	0.5% 0.5	-	-	+	-	-	-
" S	2.0% 2,0	-	-	-	+	-	-
" S	0.5% 0.5	-	-	-	+	-	-
(c) " MS	0.5% 0.0	+	-	-	+	-	-
" St	2.0% 2.0	-	-	-	+	+	-
" St	0.5% 0.5	-	-	-	+	+	-
(d) " W St	0.5% 0.0	-	+	-	+	+	-
" H	0.5% 0.5	-	-	-	+	+	+

(Table 4, Exp. 4 - continued on p.93)

TABLE 4, Experiment 4 (continued from page 92)

(a) M = Methanolic Extract

(b) W = Water Extract

(c) After extracting the wood blocks the soluble sugar content of the methanolic extract was estimated (according to Priestley, 1960) and added to the media of treatment Complete MS 0.5% as a carbon source at a concentration equivalent to 0.5% glucose.

(d) Blocks of treatment Complete W St 0.5% were first extracted with 75% methanol for 24 hours then water for a further 24 hours. Starch content of the water extract was estimated (according to Priestley, 1960) and added to the media as a carbon source at a concentration equivalent to 0.5% glucose.

Results

Experiment 1

(a) Mycelial Production. Figure 16 illustrates differences in mycelial production between the four treatments (Complete, Complete - C, Complete - N, Complete - C - N). In Figure 16A the relative amounts of growth three months after inoculation is shown while in Figure 16B the dry weight of mycelium produced in each of the treatments from two to six months after inoculation is plotted. No weights of mycelium were taken the first month after inoculation in this experiment.

In the Complete nutrient treatment maximum mycelial production is achieved shortly after inoculation, Complete - N and Complete - C - N show a gradual increase over the six month duration of the experiment while Complete - C shows little difference in production at any of the time periods.

In the presence of added carbon (treatments Complete and Complete - N) more mycelium is produced than in treatments not receiving any carbon. In comparable treatments, in the presence of carbon (Complete and Complete - N), the effect of an absence of nitrogen is a depressed mycelial production. In an absence of added carbon

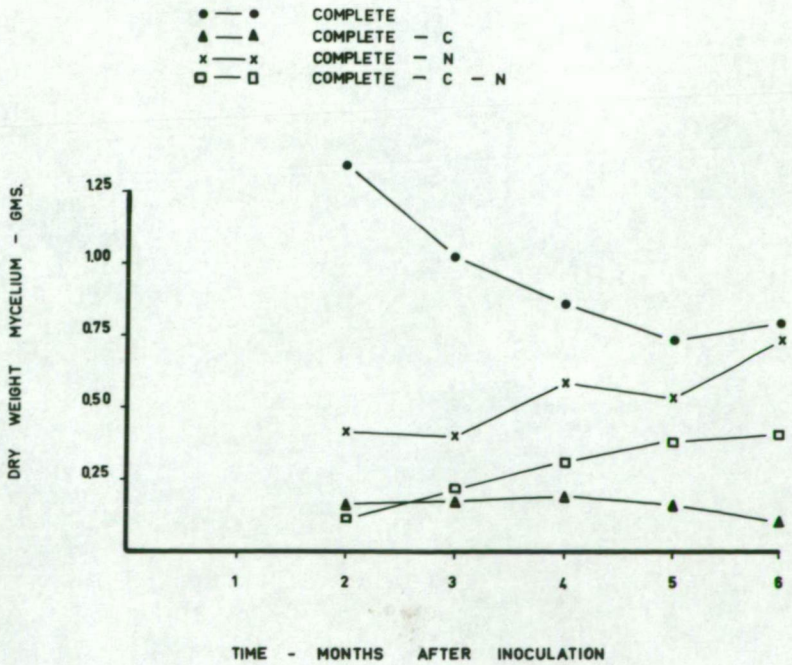
FIGURE 16

Experiment 1

16A (above) - The four treatments (Complete, Complete - C, Complete - N and Complete - C - N) three months after inoculation. The tops of the culture vessels have been cut off to show fungal growth.

16B (below) - The dry weight of mycelium produced from two to six months in the four treatments.


COMPLETE
C 2.0% N 0.5%
COMPLETE
MINUS
CARBON
COMPLETE
MINUS
NITROGEN
COMPLETE
MINUS
CARBON & NITROGEN


COMPLETE
COMPLETE – C
COMPLETE – N
COMPLETE – C – N
DRY WEIGHT MYCELIUM - GMS.
1.25
1.00
0.75
0.50
0.25
1
2
3
4
5
6
TIME - MONTHS AFTER INOCULATION

(Complete - C and Complete - C - N), an absence of nitrogen results in increased mycelial production (Figure 16B). It is difficult to explain the higher production of mycelium in Complete - C - N compared with Complete - C. It may have been expected that added nitrogen in Complete - C would have given the fungus greater ability to obtain wood carbon and so proliferate, but this has not been the case.

(b) Percent Dry Weight Loss of Wood Blocks. In Figure 17A the percent dry weight loss is plotted for the four treatments at each sampling time. Complete and Complete - N, both receiving a carbon source, show a lower loss in weight due to decay of wood by T. versicolor despite a higher production of mycelium.

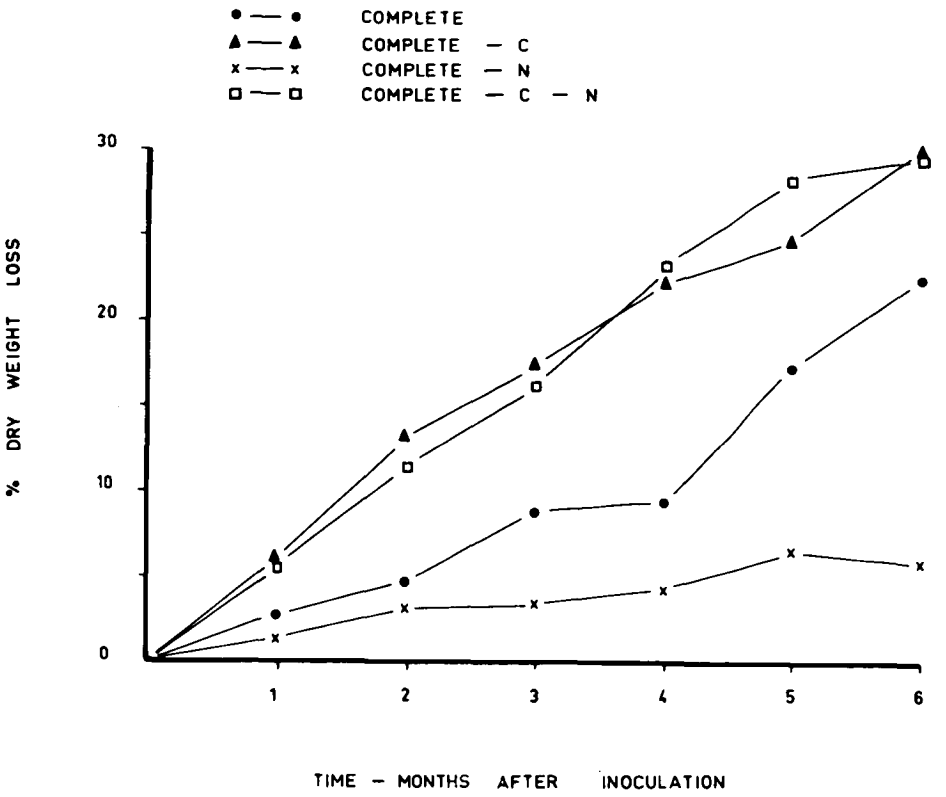
When the loss in weight of blocks is corrected for mycelial production, it can be seen from Figure 17B that the greater loss in weight of blocks in treatments not receiving carbon (Complete - C and Complete - C - N) compared with the two treatments receiving carbon (Complete and Complete - N) is accentuated. Again comparing the treatments either both receiving a carbon source (Complete and Complete - N) or an absence of carbon from both (Complete - C and Complete - C - N), the effect of an absence of nitrogen is similar in causing a slightly lower

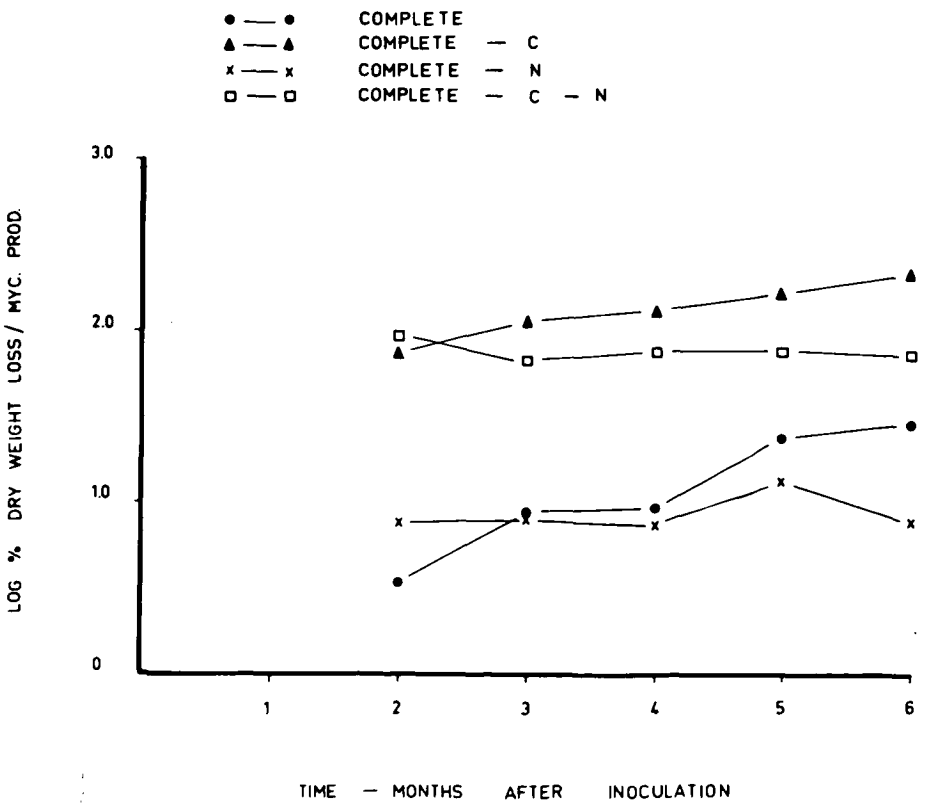
FIGURE 17

Experiment 1

17A (above) - Percent dry weight loss of wood blocks due to decay by T. versicolor. Note the higher weight loss of blocks subjected to treatments not receiving carbon.

17B (below) - Log. percent dry weight loss/mycelial production of the four treatments over the period of the experiment.


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
% DRY WEIGHT LOSS
30
20
10
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
LOG % DRY WEIGHT LOSS / MYC. PROD.
3.0
2.0
1.0
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

amount of wood decay (Figure 17B).

Some care must be taken in the interpretation of the corrected loss in weight (% dry weight loss/mycelial production) as account must be made for the influence of the agar on mycelial growth. In Complete and Complete - N growth of the mycelium extended abundantly over the surface of the media in the culture jars while in Complete - C and Complete - C - N mycelium did extend over the media but was mainly restricted to the wood blocks. (see Figure 16A).

It is felt however that legitimate use can be made of this ratio if the agar is considered to be a nutrient source (as the branch of a tree) and the wood block material being subjected to fungal attack under different nutrient conditions.

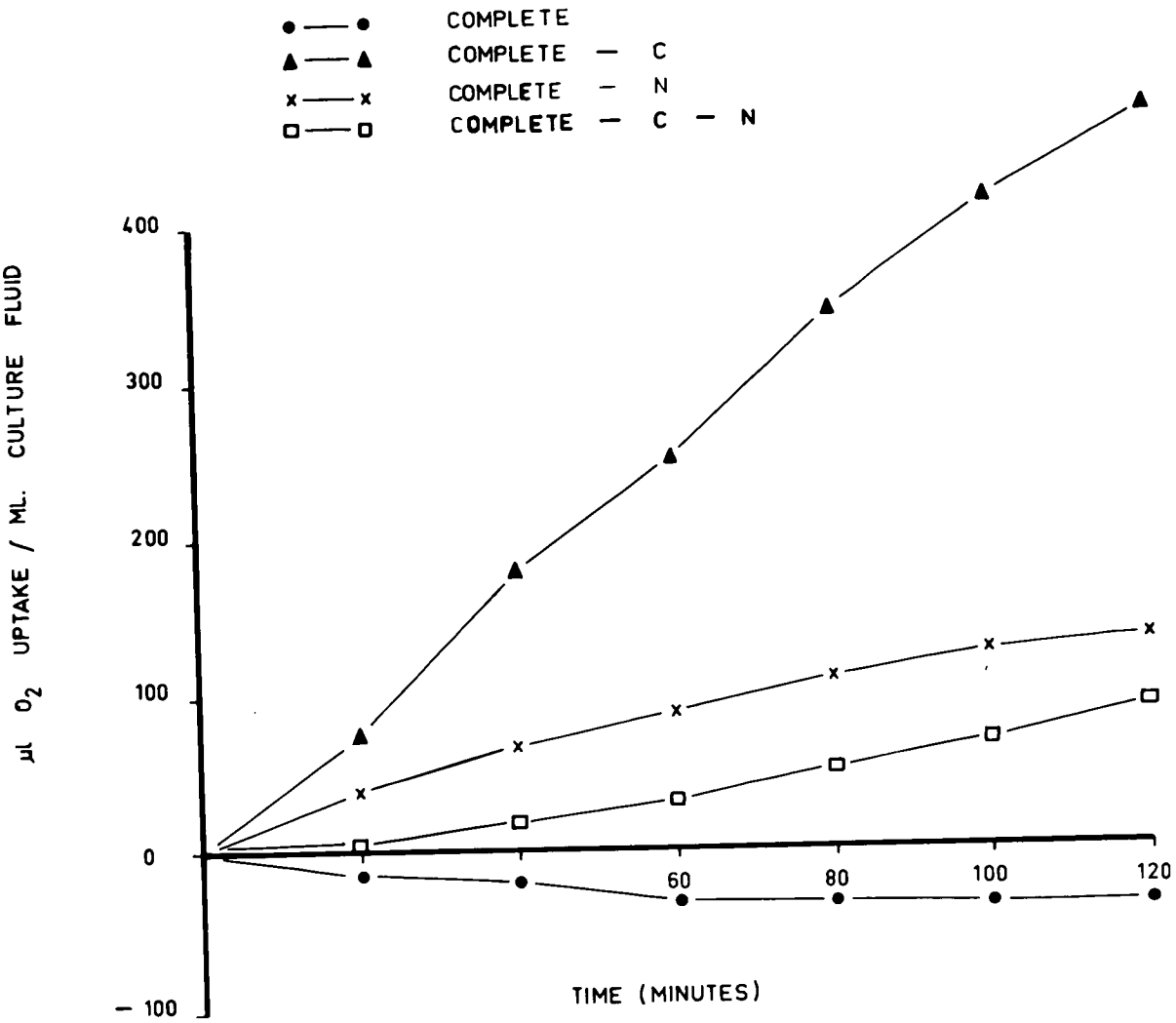
Experiment 1A

The extracellular polyphenol oxidase production by T. versicolor was estimated after growing the fungus on wood in solution culture. The results of this bioassay technique can be seen in Figure 18. In the absence of any carbon supply in Complete - C, (except that present in the wood) a considerable amount of enzyme was produced. Treatments Complete - N and Complete - C - N produced lower

FIGURE 18

Experiment 1A

The oxidase production by T. versicolor expressed in terms of µl O_2 uptake. Note the much higher levels of enzyme produced in the treatment Complete - C compared with the Complete nutrient treatment.


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
400
300
200
100
0
— 100
µl O_2 UPTAKE / ML. CULTURE FLUID
60
80
100
120
TIME (MINUTES)

amounts of enzyme, while in the presence of adequate amounts of carbon and nitrogen sources no enzyme was produced. In the two treatments not receiving a carbon supply (Complete - C and Complete - C - N), an absence of nitrogen resulted in a much lower amount of enzyme produced (Figure 18). This effect may have been anticipated and is reflected in the dry weight loss due to decay by T. versicolor, when in the absence of nitrogen (Complete - C - N) a lower relative amount of decay is recorded (Figure 17B). However in treatments receiving a carbon supply (Complete and Complete - N) an absence of nitrogen resulted in a higher production of enzyme by the fungus (Figure 18) but not a greater amount of wood decay (Figure 17B).

Experiment 2

This experiment was conducted substituting Bacto-Peptone, as used in experiment 1, with $(NH_4)_2HPO_4$ to determine if the carbon content of Bacto-Peptone and the use of an inorganic nitrogen source would in any way influence the results obtained from the first experiment. In addition, for treatments receiving glucose, the glucose level was raised to 3.0% and 10 mgms. of yeast was added to all treatments to enhance an earlier establishment of the fungus to reduce the risk of contamination of culture vessels.

(a) Mycelial Production. The use of an inorganic nitrogen source confined all the growth of Complete - C to the wood block (Figure 19A) compared with the same treatment in experiment 1 (Figure 16A). This may have been in part due to the absence of any carbon in the media and the fungus had to rely entirely on the wood block as a carbon source. This explanation however, does not account completely for the absence of mycelial growth in this treatment because Complete - C - N supported some growth on its media. The only difference between these two treatments was the presence of 0.5% nitrogen in the media of Complete - C, which must also have contributed to the confinement of mycelial growth to the wood block. Mycelial growth in Complete - C was depressed in this experiment (Figure 19B) compared with experiment 1 (Figure 16B).

The mycelial production of Complete - C - N (Figure 19B) remained the same as in experiment 1 (Figure 16B), which was to be expected as the media in both experiments remained unchanged except for the addition of 10 mgms. of yeast in experiment 2.

In Complete and Complete - N, growth was generally higher than in the previous experiment (c.f. Figure 16B and Figure 19B) due to the addition of yeast and higher media glucose levels.

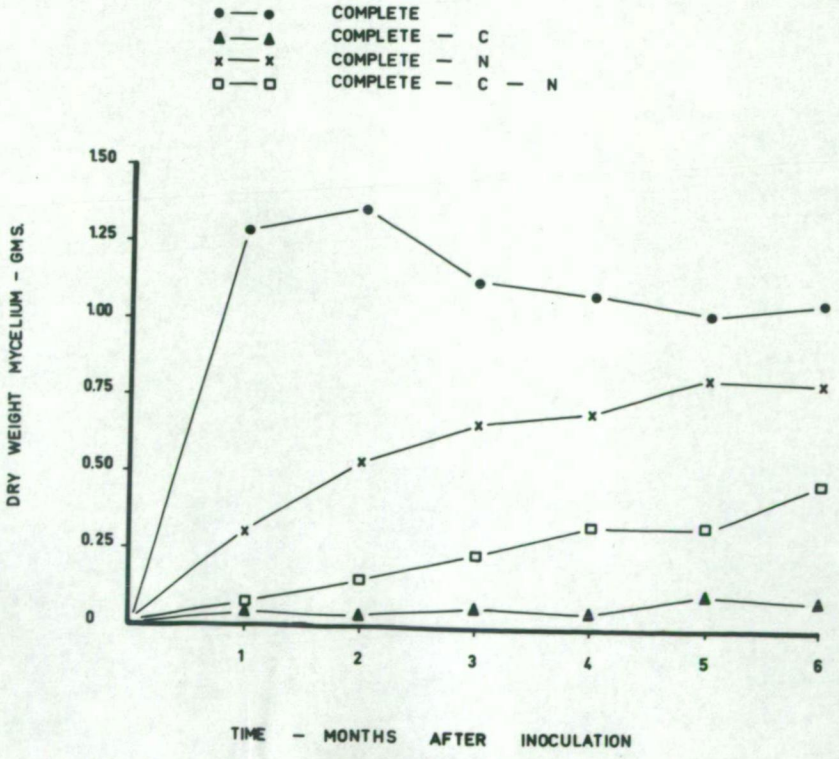
FIGURE 19

Experiment 2

19A (above) - The four treatments Complete, Complete - C, Complete - N and Complete - C - N two months after inoculation. Note the absence of mycelial growth on the media of Complete - C.

19B (below) - The dry weight of mycelium produced over the six month duration of the experiment.


COMPLETE
C 3% N 0.5%
COMPLETE
MINUS
CARBON
COMPLETE
MINUS
NITROGEN
COMPLETE
MINUS
CARBON & NITROGEN


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
DRY WEIGHT MYCELIUM - GMS.
1.50
1.25
1.00
0.75
0.50
0.25
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

(b) Percent Dry Weight Loss. The depressed growth of mycelium in Complete - C caused the actual weight loss to be at a lower level (Figure 20A) than in the first experiment (Figure 17A). However when correction is made for mycelial production the relative weight loss is similar in both experiment 1 (Figure 17B) and experiment 2 (Figure 20B). As with mycelial production the weight loss of blocks in Complete - C - N show little difference between the two experiments (Figure 17A and 17B and Figure 20A and 20B). Complete showed an overall lower loss in weight (Figure 17A and Figure 20A) despite a higher mycelial production (Figure 16B and Figure 19B). In Complete - N similar trends to Complete were seen with lower loss in weight (Figure 17A and Figure 19A) accompanied by a higher production of mycelium (Figure 16B and Figure 19B).

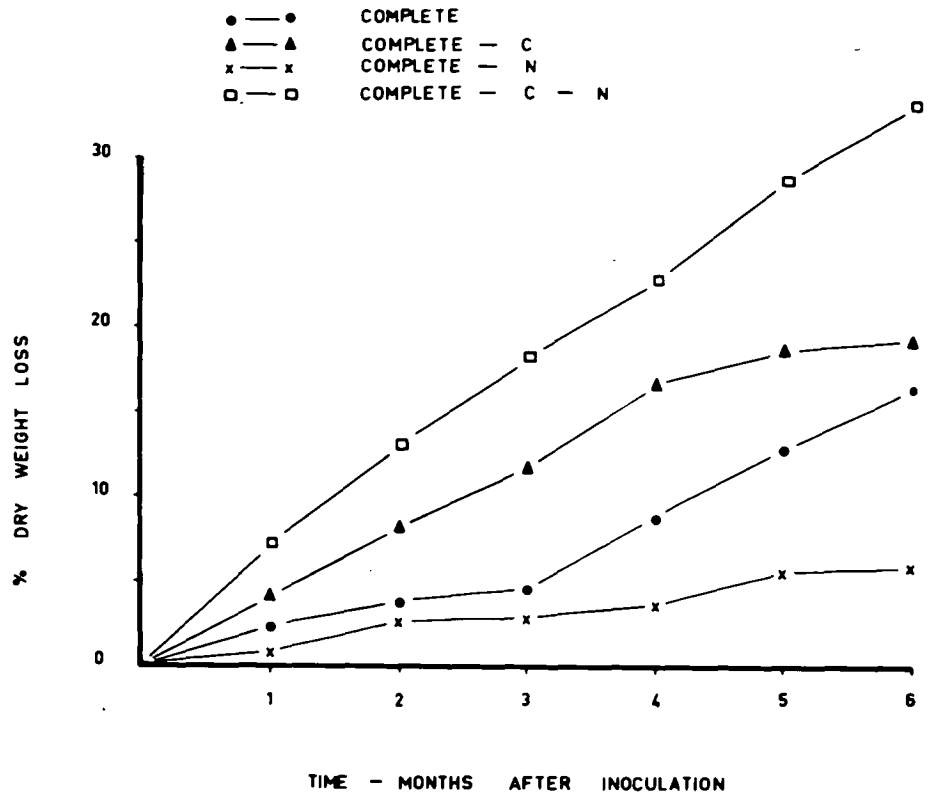
The effects of the nitrogen and sugar supplies remained the same in this experiment as experiment 1. The presence of glucose consistently suppressed wood decay but nitrogen did not have this effect. Results from both experiments 1 and 2 indicated that sugars play a more direct role in limiting the decay of wood by T. versicolor than nitrogen.

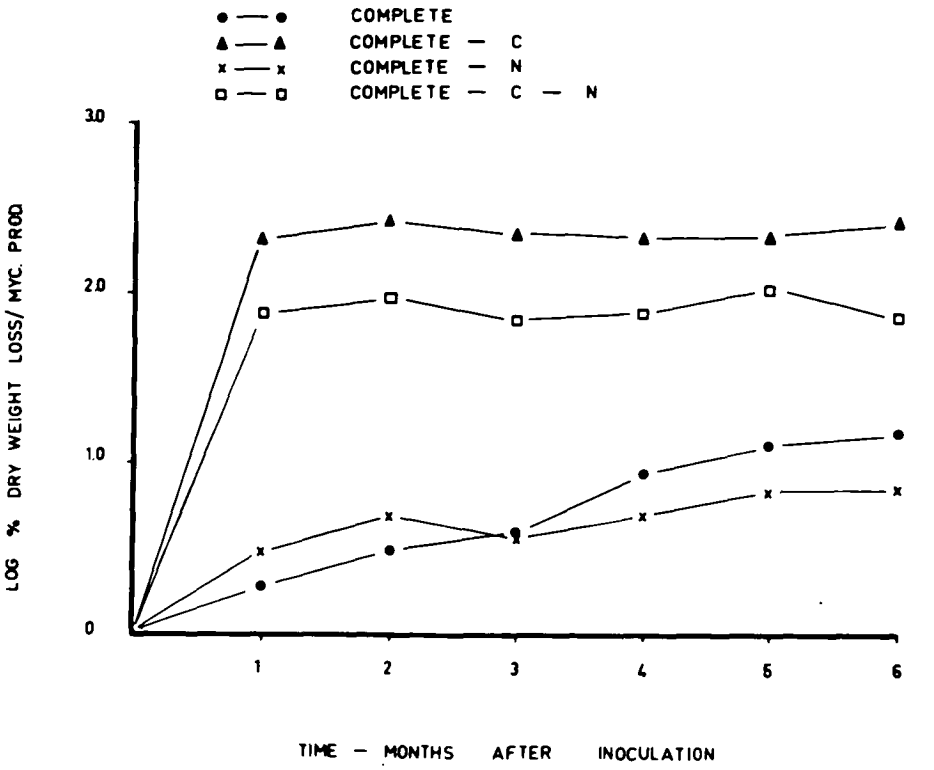
FIGURE 20

Experiment 2

20A (above) - Percent loss in weight of wood blocks subjected to attack by T. versicolor grown under different nutrient conditions.

20B (below) - Log. Percent dry weight loss/mycelial production for the four treatments over the six month duration of the experiment.


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
% DRY WEIGHT LOSS
30
20
10
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION


COMPLETE
COMPLETE — C
COMPLETE — N
COMPLETE — C — N
LOG % DRY WEIGHT LOSS/ MYC. PROD
3.0
2.0
1.0
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

Experiment 3

It was established in experiments 1 and 2 that adequate levels of carbon were required in the media contained in the culture vessels to reduce the amount of wood decay when blocks were subjected to different nutrient treatments. Because of this and the very large amounts of carbohydrates required by a bearing apple tree, it was decided to investigate the effects of different levels of glucose on the development of wood decay by T. versicolor.

As mentioned in the Method (Part D) six different levels of glucose were used, these being 3.0%, 2.0%, 1.5%, 1.0%, 0.5% and 0.0%. The two levels 3.0% and 0.0% were the same treatments as Complete and Complete - C in experiment 2.

(a) Mycelial Production. Production of mycelium in this experiment is shown in Figure 21A and 21B. The amount of mycelium produced increases in direct proportion with increasing glucose levels. Maximum quantity of mycelium is, with the exception of Complete 0.0%, produced after the first month. Treatments receiving 3.0%, 2.0%, and 1.5% show a slight drop after the first month to a constant level. Complete 1.0% and 0.5% maintain a constant amount of mycelium throughout the experiment while the treatment receiving no glucose produces little mycelium but tends to increase after six months.

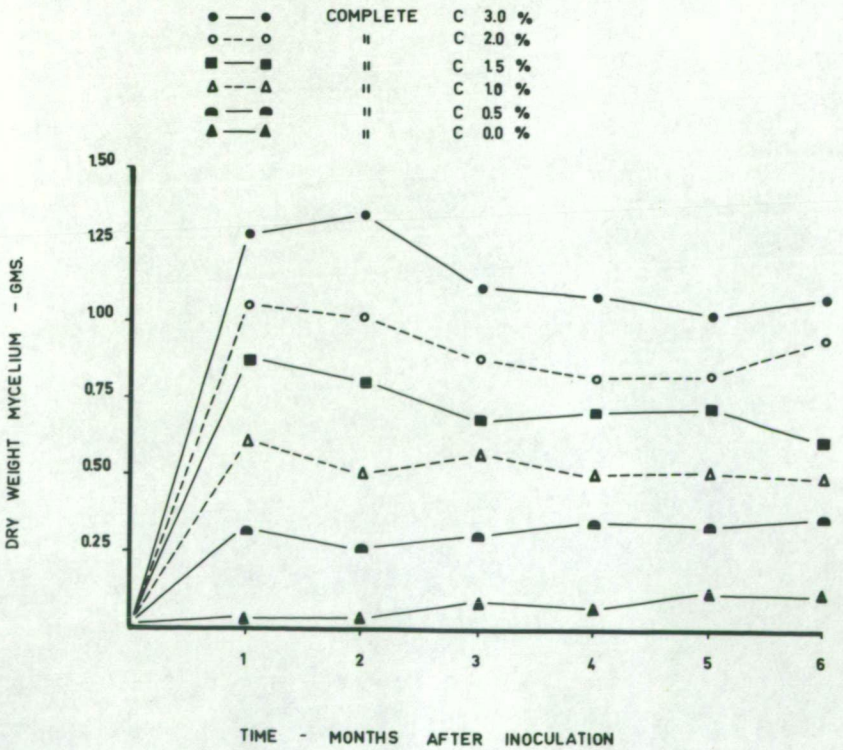
FIGURE 21

Experiment 3

21A (above) - Illustrating four levels of glucose and their effects on the growth of mycelium. See Figure 19A for treatments receiving 3.0% and 0.0% glucose.

21B (below) - The dry weight of mycelium produced by six treatments receiving different levels of glucose.


COMPLETE
C 0.5% N 0.5%
COMPLETE
C 1.0% N 0.5%
COMPLETE
C 1.5% N 0.5%
COMPLETE
C 2.0% N 0.5%


COMPLETE C 3.0 %
" C 2.0 %
" C 1.5 %
" C 1.0 %
" C 0.5 %
" C 0.0 %
DRY WEIGHT MYCELIUM - GMS.
1.50
1.25
1.00
0.75
0.50
0.25
1
2
3
4
5
6
TIME - MONTHS AFTER INOCULATION

(b) Percent Dry Weight Loss. With the exception of Complete 0.0% the percent dry weight loss shows a close inverse relation with the glucose levels (Figure 22A) and hence the production of mycelium. This relation is seen more clearly in Figure 22B when the correction for mycelial production has been made. With increasing sugar levels dry weight loss decreases.

Figures 23A, 23B and 24 compare mycelial production, dry weight loss and percent dry weight loss/mycelial production respectively with sugar level and time. Figure 23A demonstrates the increase in mycelial production with increasing levels of sugar but little time effect. An increase in decay with decreasing levels of sugar and increasing time is shown in Figure 23B. In Figure 24 where account has been made for mycelial production the inverse relation of sugar level and percent dry weight loss is seen with the influence of time becoming less important as the sugar levels decrease. This Figure summarises the results from experiment 3.

Experiment 3A

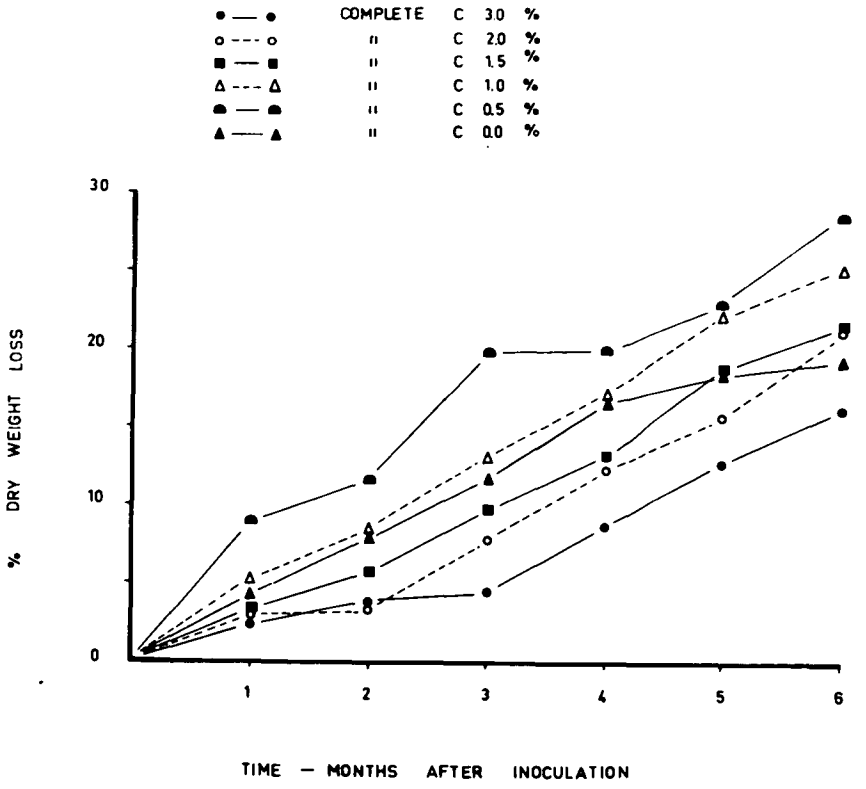
The production of extracellular polyphenol oxidase by the fungus grown on wood in solution culture containing different sugar levels is illustrated in Figure 25.

FIGURE 22

Experiment 3

22A (above) - Percent loss in weight of wood blocks subjected to attack by T. versicolor when grown in the presence of six different levels of glucose.

22B (below) - Log. Percent dry weight loss/mycelial production for treatments receiving six different levels of glucose.


COMPLETE C 3.0 %
" C 2.0 %
" C 1.5 %
" C 1.0 %
" C 0.5 %
" C 0.0 %
% DRY WEIGHT LOSS
30
20
10
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

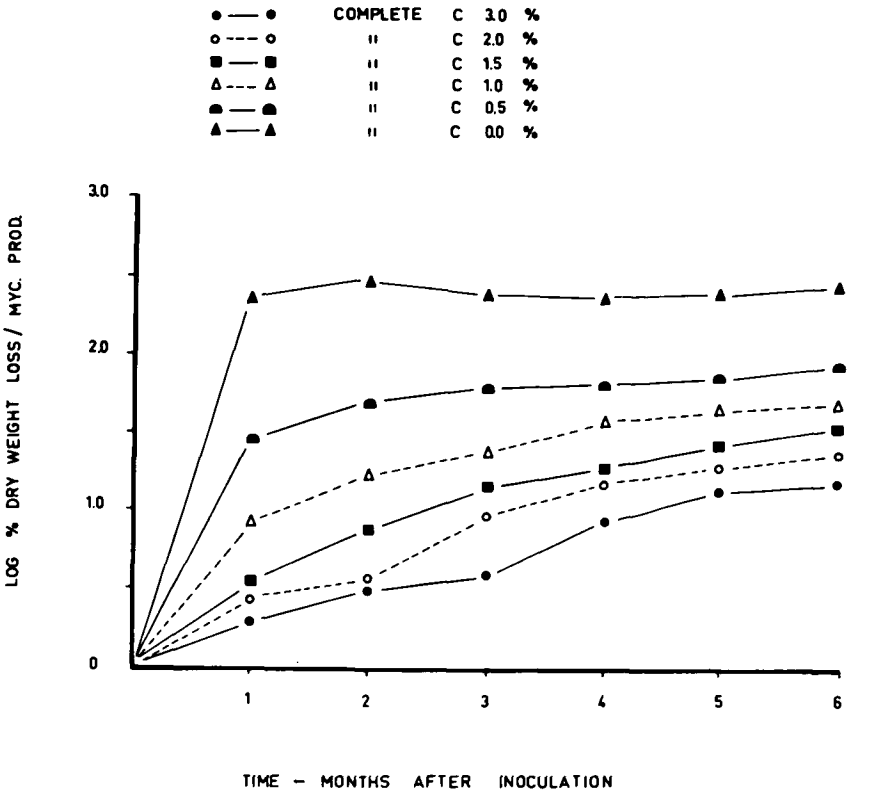
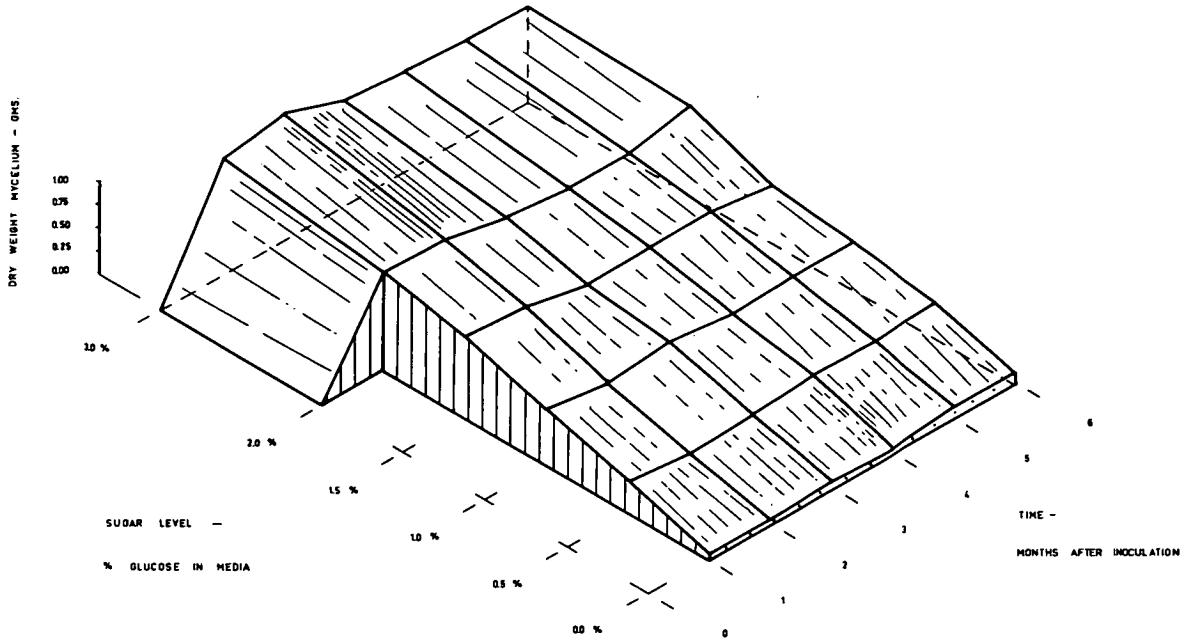

COMPLETE C 3.0 %
" C 2.0 %
" C 1.5 %
" C 1.0 %
" C 0.5 %
" C 0.0 %
LOG % DRY WEIGHT LOSS / MYC. PROD.
3.0
2.0
1.0
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

FIGURE 23

Experiment 3

23A (above) - Mycelial production by T. versicolor over six months when grown on media containing different glucose levels.

23B (below) - Percent dry weight loss of blocks over six months when subjected to attack by T. versicolor grown on media containing different glucose levels.


DRY WEIGHT MYCELIUM - GMS.
1.00
0.75
0.50
0.25
0.00
3.0 %
2.0 %
1.5 %
1.0 %
0.5 %
0.0 %
SUGAR LEVEL —
% GLUCOSE IN MEDIA
0
1
2
3
4
5
6
TIME -
MONTHS AFTER INOCULATION

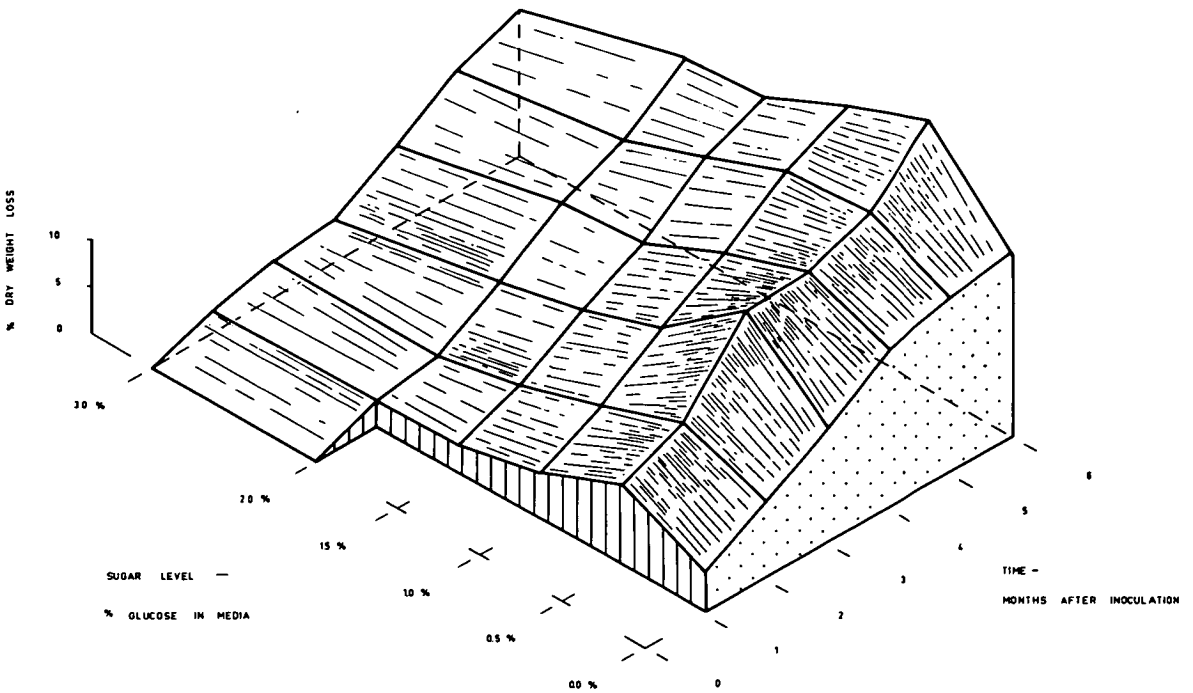

% DRY WEIGHT LOSS
10
5
0
3.0 %
2.0 %
1.5 %
1.0 %
0.5 %
0.0 %
SUGAR LEVEL —
% GLUCOSE IN MEDIA
0
1
2
3
4
5
6
TIME -
MONTHS AFTER INOCULATION

FIGURE 24

Experiment 3

Log. percent dry weight loss/mycelial production - Relative weight loss of blocks when subjected to attack by T. versicolor grown in the presence of different levels of glucose over a period of six months.

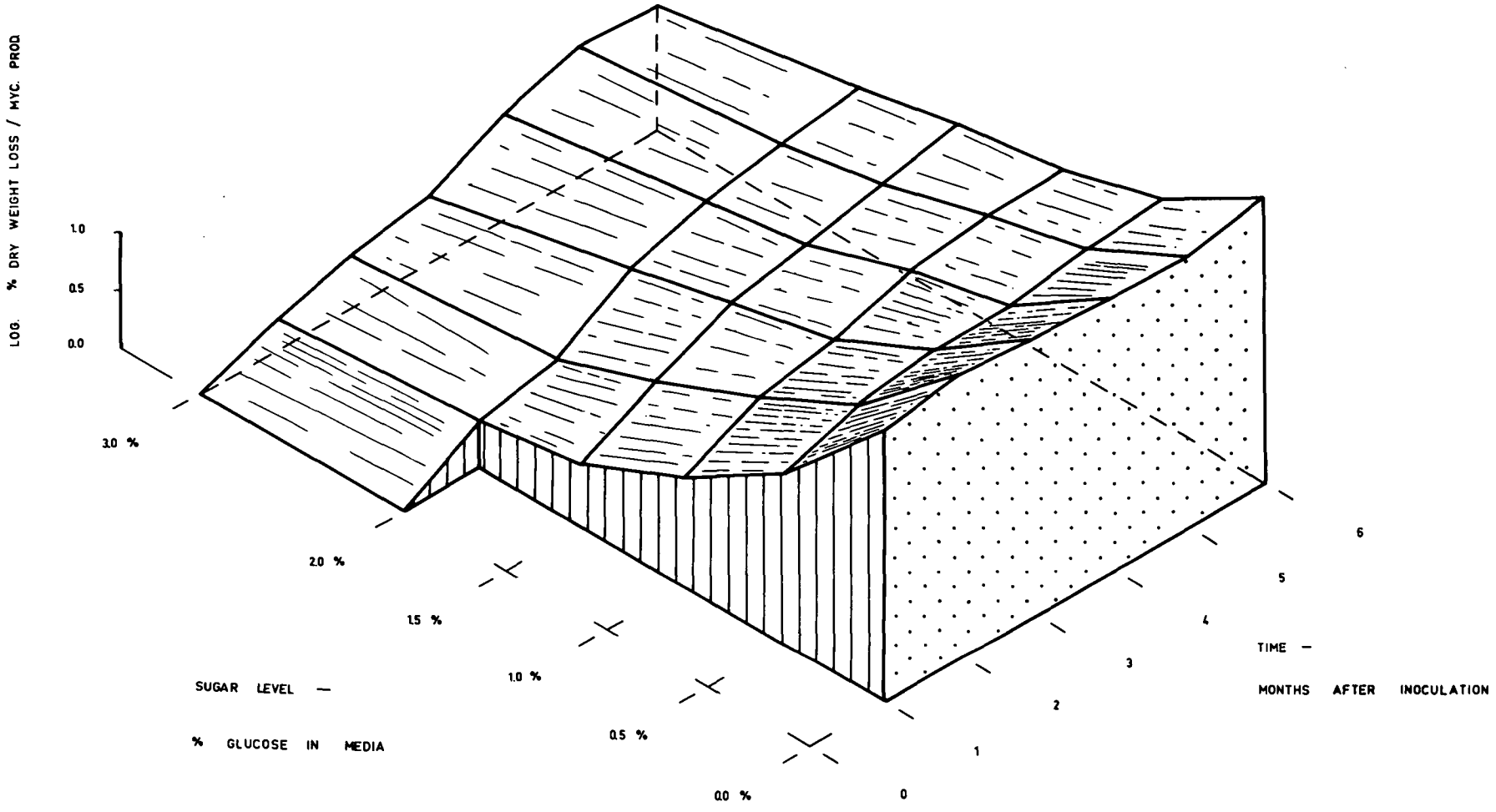
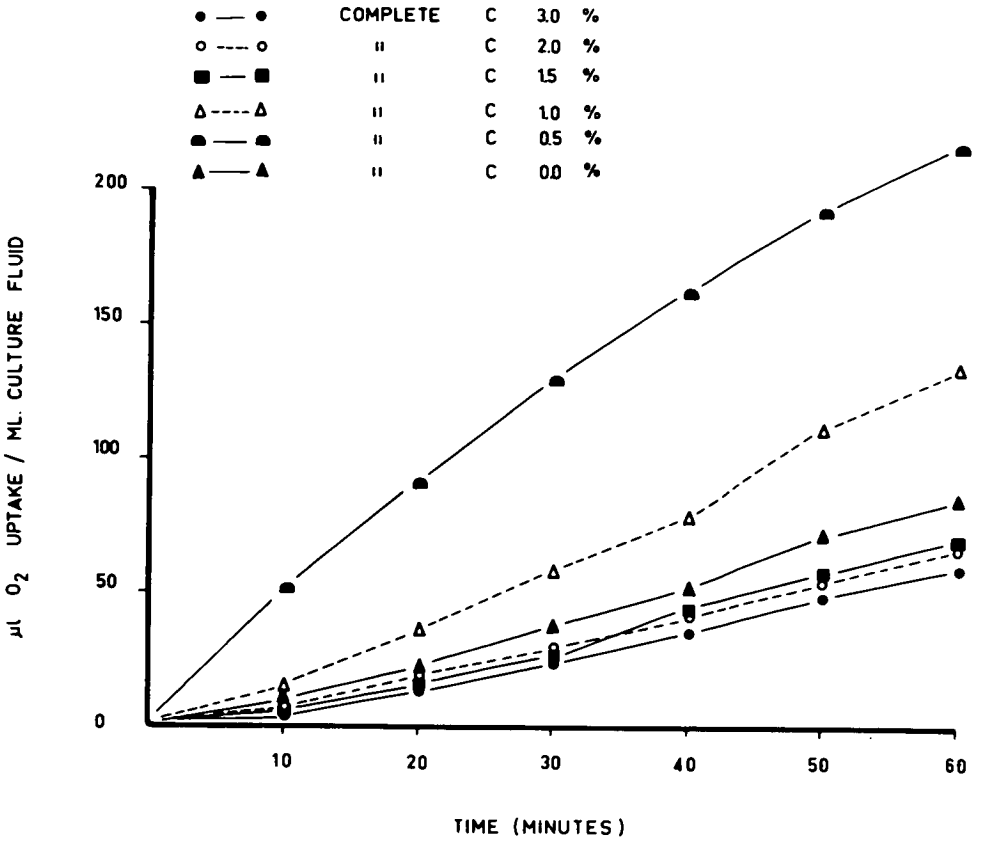

LOG. % DRY WEIGHT LOSS / MYC. PROD.
1.0
0.5
0.0
3.0 %
2.0 %
1.5 %
1.0 %
0.5 %
0.0 %
SUGAR LEVEL —
% GLUCOSE IN MEDIA
0
1
2
3
4
5
6
TIME —
MONTHS AFTER INOCULATION

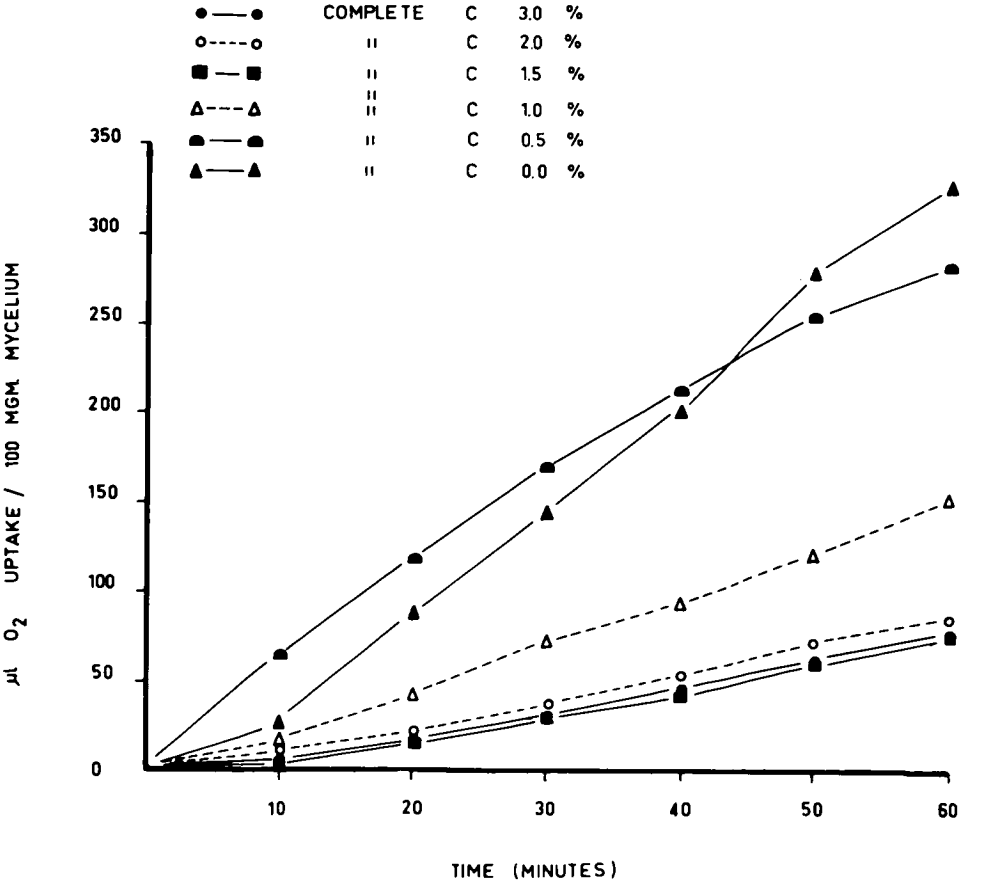
FIGURE 25

Experiment 3A

25A (above) - The polyphenol oxidase production per ml. of culture fluid by T. versicolor when grown in the presence of six different glucose levels.

25B (below) - The polyphenol oxidase production per ml. of culture fluid per 100 mgm. of mycelium produced by T. versicolor when grown in the presence of six different glucose levels.


COMPLETE C 3.0 %
" C 2.0 %
" C 1.5 %
" C 1.0 %
" C 0.5 %
" C 0.0 %
µl O2 UPTAKE / ML. CULTURE FLUID
0
50
100
150
200
10
20
30
40
50
60
TIME (MINUTES)


COMPLETE C 3.0 %
" C 2.0 %
" C 1.5 %
" C 1.0 %
" C 0.5 %
" C 0.0 %
µl O2 UPTAKE / 100 MGM. MYCELIUM
0
50
100
150
200
250
300
350
10
20
30
40
50
60
TIME (MINUTES)

Glucose added to the media at concentrations of 3.0%, 2.0% and 1.5% suppressed enzyme production to the same extent, 1.0% induced considerable suppression but not as much as the higher levels while 0.5% or an absence of glucose had little suppressing influence.

Experiment 4

To determine if soluble sugars and starches contained in the wood blocks in any way affected the ability of apple wood to withstand attack by T. versicolor, wood blocks were:

(a) untreated;

(b) extracted for 24 hours with 75% aqueous methanol to remove soluble materials;

(c) extracted for 24 hours with 75% aqueous methanol, 8 hours with acidified methanol and 8 hours with 96% methanol, then water for a further 24 hours to remove both soluble materials and starches;

(d) extracted with methanol and water then hydrolyzed with N H_2SO_4 to remove hemicelluloses.

The blocks so treated were then subjected to attack by T. versicolor grown on media containing either 2.0% or 0.5% sucrose. These levels of sugar were chosen because (i) 2.0% sugar had been shown to suppress both enzyme production

and decay while promoting mycelial growth (Experiment 3) and (ii) 0.5% sugar did not suppress enzyme production by the fungus and at the same time promoted mycelial growth to an extent where vigorous attack of the wood occurred.

Two other treatments - (i) Complete MS 0.5% where the methanolic extract was added to the media as a carbon source to give a soluble sugar concentration of 0.5% glucose equivalents and (ii) Complete WSt 0.5% where the water extract was added to the media as a carbon source to give a starch concentration equivalent to 0.5% glucose - were included to determine the effects of these wood fractions on the growth of the fungus and their ability to suppress fungal decay of wood.

Table 5 includes average pre- and post- extraction weights of blocks used in this experiment plus sugar and starch levels expressed as an average on a percent dry weight basis for blocks in the various treatments.

A. Treatments receiving 2.0% sucrose added to the media

These included Complete C 2.0% where wood blocks were untreated, Complete S 2.0% where wood blocks were extracted with methanol and Complete St 0.5% in which wood

TABLE 5 (a)

	Treatment								
	Complete								
	C 2.0%	C 0.5%	S 2.0%	S 0.5%	MS 0.5%	St 2.0%	St 0.5%	WSt 0.5%	H 0.5%
Average weight of original block (gms.)	5.44 (b)	5.58 (b)	5.75	5.62	5.54	6.02	5.42	5.77	+
Average weight of extracted blocks (gms)	-	-	5.55	5.46	5.37	5.58	5.14	5.30	2.92 (b)
Average % loss in weight due to 24 hr. extraction with 75% methanol	-	-	3.42	2.79	3.13	+	+	+	+
Average % loss in weight due to 24 hr. extraction with 75% methanol followed by 24 hr. extraction with water	-	-	+	+	+	7.33	5.44	8.15	+

(continued on page 115)

Table 5 (a) (continued from page 114)

	Treatment								
	Complete								
	C 2.0%	C 0.5%	S 2.0%	S 0.5%	MS 0.5%	St 2.0%	St 0.5%	WSt 0.5%	H 0.5%
Average % level soluble sugar (methanol extract) in wood expressed as glucose equivalents	+	+	0.96	0.78	0.95	0.31	1.25	0.61	+
Average % level starch (Water extract) in wood expressed as glucose equivalents	+	+	+	+	+	2.75	1.19	3.43	+
% loss in weight due to extraction with methanol not attributable to soluble sugars	-	-	2.46	2.01	2.18	+	+	+	+
% loss in weight due to extraction with methanol & water not attributable to soluble sugars or starches	-	-	+	+	+	4.27	3.00	4.11	+

+ No figures available
(a) All figures represent an average of 40 blocks unless otherwise indicated.
(b) Average of 24 blocks

blocks were extracted with methanol and water before being subjected to attack by the fungus.

(a) Mycelial Production. Complete C 2.0% produced a maximum amount of mycelium by the end of the first month (Figure 26A). This level was maintained until the end of the second month which was followed by a gradual reduction in the amount of mycelium present to a constant level approximately 0.4 gms. below the initial maximum. The removal of the fraction containing the soluble sugars (Complete S 2.0%) resulted in a delay of one month compared with the previous treatment, to achieve maximum mycelial growth. Maximum growth was then maintained over the 2-3 month period rather than 1-2 as in Complete C 2.0% (Figure 26A). A similar reduction in the amount of mycelium present occurred, as in Complete C 2.0%, to a level approximately 0.4 gms. below the maximum.

By extracting blocks with both methanol and water (Complete St 2.0%), although a maximum production of mycelium was reached after the second month which was equivalent to that produced in the two previous treatments, this was not maintained and an immediate decline in the amount of mycelium present occurred.

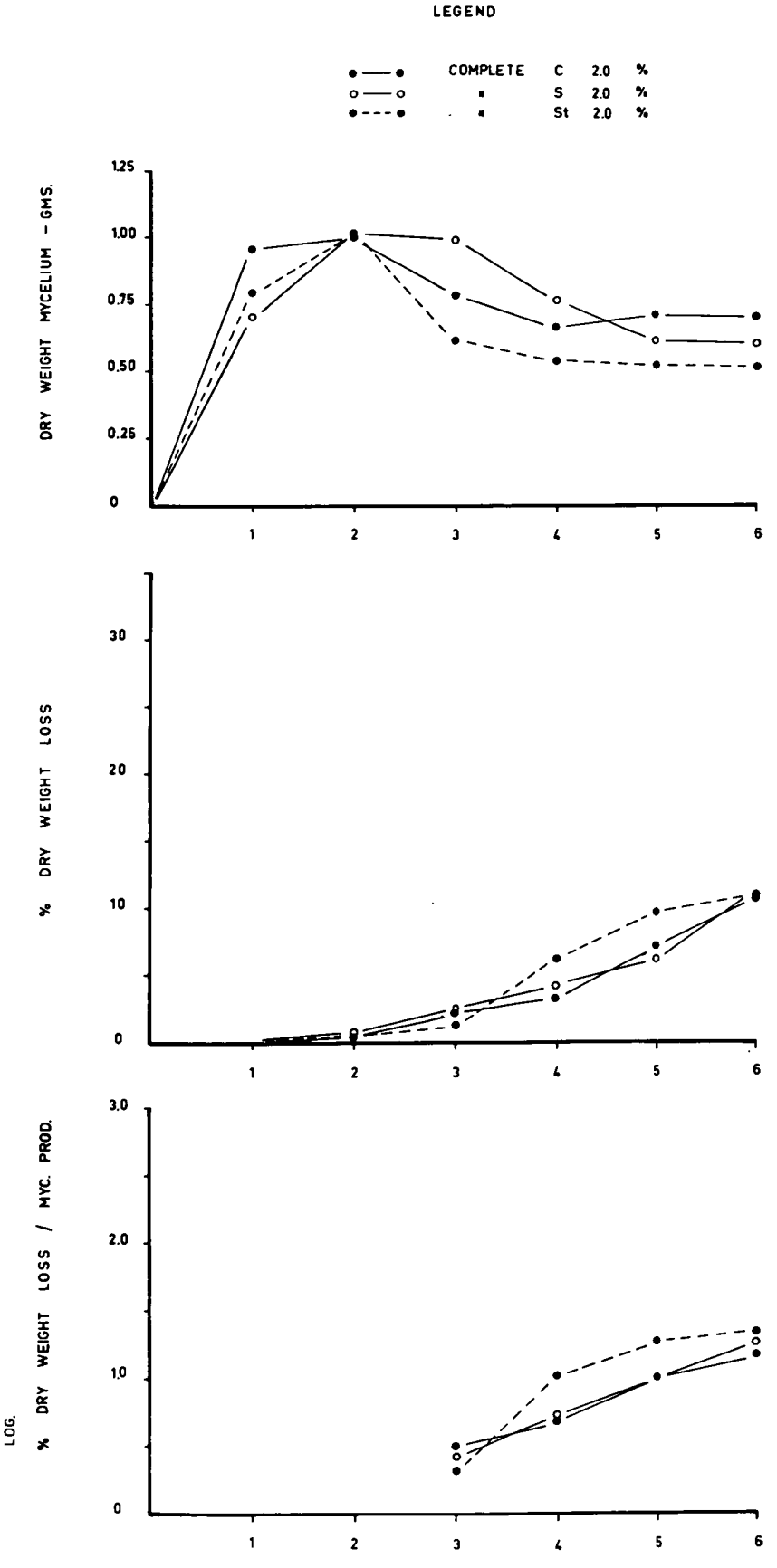
FIGURE 26

Experiment 4

26A (above) - Mycelial production for treatments Complete C 2.0%, Complete S 2.0% and Complete St 2.0%.

26B (centre) - Percent dry weight loss of treatments Complete C 2.0%, Complete S 2.0% and Complete St 2.0%.

26C (below) - Log. Percent dry weight loss/mycelial production for treatments Complete C 2.0%, Complete S 2.0% and Complete St 2.0%.


LEGEND
COMPLETE C 2.0 %
S 2.0 %
St 2.0 %
DRY WEIGHT MYCELIUM - GMS.
1.25
1.00
0.75
0.50
0.25
0
1
2
3
4
5
6
% DRY WEIGHT LOSS
30
20
10
0
LOG. % DRY WEIGHT LOSS / MYC. PROD.
3.0
2.0
1.0
0
TIME - MONTHS AFTER INOCULATION

The initial maximum level in the three treatments may be attributable to the high sugar levels of the media on which the fungus was grown. The first extraction of blocks with methanol has however, caused a delay by one month in achieving maximum fungal growth. The materials contained in the methanolic extractable fraction of the wood have then contributed in some way to the growth of the fungus.

The maintenance of maximum mycelial growth in Complete C 2.0% and Complete S 2.0% could be explained in terms of the starch content of the wood. Sufficient starch may have been present in the wood to maintain the high level of mycelium produced through the stimulation of media sugar. After wood starch was exhausted such a level could not be maintained by readily available, soluble forms of carbon.

In the absence of any readily available soluble materials or starch in the wood of Complete St 2.0%, although an initial maximum growth was achieved because of high media sugar levels this amount of growth could not be readily supported by available substances in the wood and an immediate drop in the amount of mycelium present occurred.

(b) % Dry Weight loss. Comparing the effect of the presence of 2.0% sucrose in the media of treatments Complete C 2.0% and Complete S 2.0%, it can be seen from Figure 26B and 26C, that no influence on the percent loss in weight of blocks due to fungal decay resulted from the removal of the methanolic fraction from the wood. Complete St 2.0% had higher amounts of decay initially but approached the level of the other two treatments by the end of six months.

The lack of decay in the three treatments up to the end of three months (Figure 26B and 26C) suggests a suppression of fungal enzymes by media sugar and soluble wood carbohydrates in Complete C 2.0% and Complete S 2.0% and media sugar in Complete St 2.0%. An apparent delay in the exhaustion of these sources to be expressed in an increase in decay is implied if the explanation for mycelial growth is to be accepted.

According to the explanation of mycelial growth no starch resources remain in the wood of treatments Complete C 2.0% and Complete S 2.0% by the end of three months. It is at this time that the percent loss in weight due to decay commences to increase (Figure 26B and 26C). The loss in weight of blocks subjected to Complete St 2.0% increases

at the end of three months but at a higher rate than either of the other treatments.

B, Treatments receiving 0.5% sucrose in media.

In addition to blocks being untreated, extracted with methanol and extracted with methanol and water, a further treatment was included where blocks were first extracted with methanol and water then hydrolyzed with N H_2SO_4 to remove hemicelluloses (Complete H 0.5%) before being subjected to attack by T. versicolor grown on a media containing 0.5% sucrose.

(a) Mycelial production. Unlike the treatments receiving 2.0% sucrose, the removal of the methanolic fraction from the wood before subjecting blocks to decay by the fungus (Complete S 0.5%) resulted in an increased production of mycelium compared with Complete C 0.5% (Figure 27A). This suggests than an inhibitory factor to the growth of the fungus had been removed. In the previous treatments this had evidently been masked by the high levels of sugar in the media.

Complete C 0.5% and Complete St 0.5% displayed similar growth patterns (Figure 27A) despite the removal

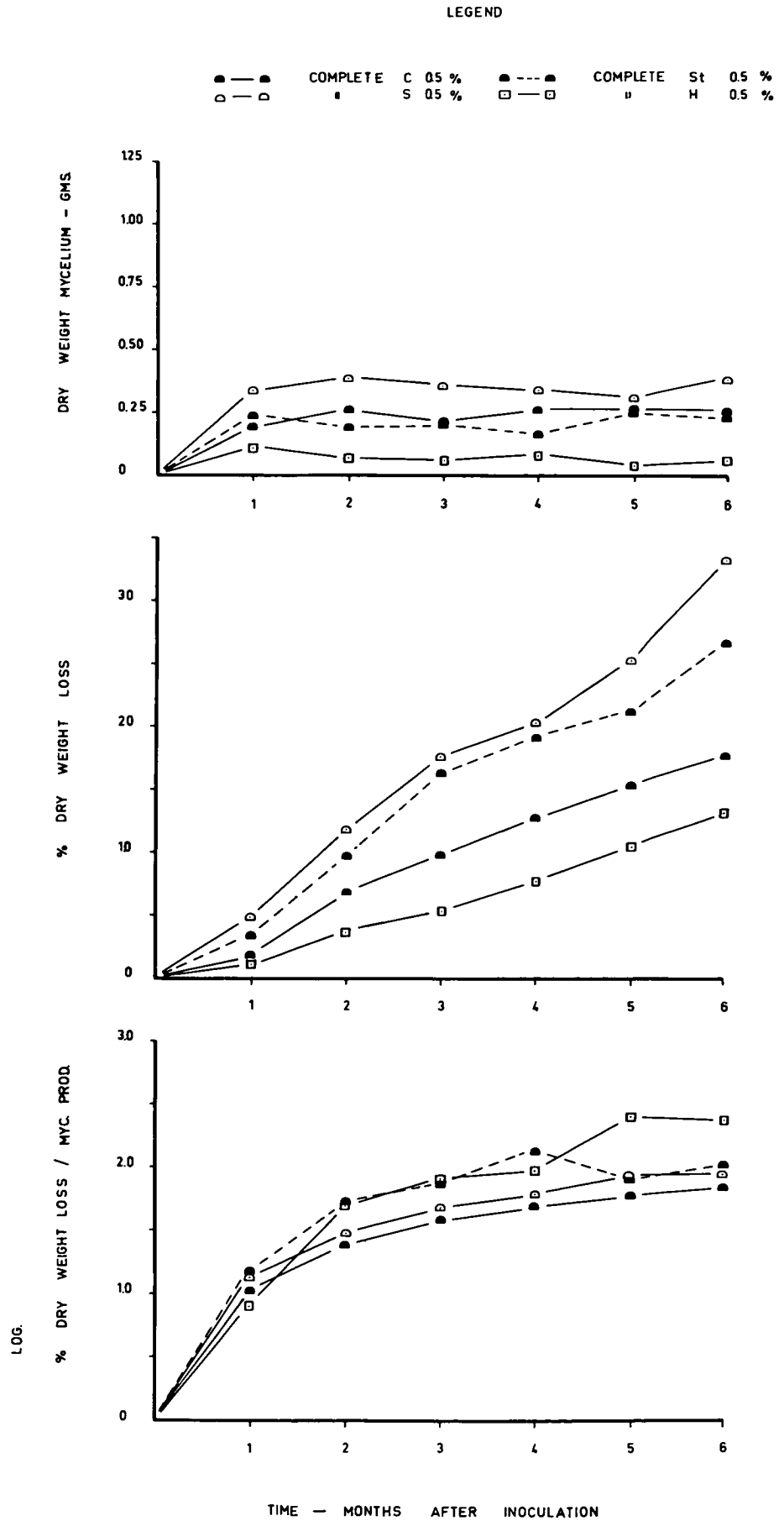
FIGURE 27

Experiment 4

27A (above) - Mycelial production for treatments Complete C 0.5%, Complete S 0.5%, Complete St 0.5% and Complete H 0.5%.

27B (centre) - Percent dry weight loss due to decay by T. versicolor in treatments Complete C 0.5%, Complete S 0.5%, Complete St 0.5% and Complete H 0.5%.

27C (below) - Log. percent dry weight loss/mycelial production in treatments Complete C 0.5%, Complete S 0.5%, Complete St 0.5% and Complete H 0.5%.


LEGEND
COMPLETE C 0.5 %
" S 0.5 %
COMPLETE St 0.5 %
" H 0.5 %
DRY WEIGHT MYCELIUM - GMS.
125
1.00
0.75
0.50
0.25
0
1
2
3
4
5
6
% DRY WEIGHT LOSS
30
20
10
0
% DRY WEIGHT LOSS / MYC. PROD.
LOG.
3.0
2.0
1.0
0
TIME — MONTHS AFTER INOCULATION

of the proposed inhibitory factor in Complete St 0.5%. Fungal growth could not however rely on soluble wood carbon as a readily available carbon source in the latter treatment.

In Complete H 0.5% very little growth of mycelium occurred, suggesting, when comparing this treatment with Complete St 0.5% that the fungus is able to use the hemicellulose fraction to produce approximately twice as much growth in Complete St 0.5% than in Complete H 0.5% (Figure 27A).

Results from mycelial growth in these treatments suggest an inhibitory factor to the growth of the fungus present in the methanolic fraction of the wood extract and the inability of the fungus to become established in the absence of a comparatively soluble form of carbon other than that supplied in the media.

(b) Percent dry weight loss. Little difference is evident in the actual percent dry weight loss in treatments Complete S 0.5% and Complete St 0.5% and both show higher amounts of loss than Complete C 0.5% (Figure 27B). Actual weight loss in Complete H 0.5% is at a lower level than Complete C 0.5%.

However when correction is made for the different amounts of mycelium produced (Figure 27C), it is seen that Complete C 0.5% and Complete S 0.5% have in fact approximately the same relative weight loss, the difference in actual weight loss being a function of the mycelium produced.

As in the treatments receiving 2.0% sucrose in the media, Complete St 0.5% has a relative weight loss (Figure 27C) initially higher than either Complete C 0.5% or Complete S 0.5% but after six months no difference exists between the three treatments.

Complete H 0.5%, in the absence of any soluble wood carbon, has the greatest relative weight loss of all treatments. Interpretation of this result must be done with some caution as the extracted weight of blocks used was approximately half that of blocks used in other treatments (see Table 5).

C. Treatments receiving methanol or water extracts added to the media as a carbon source at a concentration of 0.5% glucose equivalents.

(a) Mycelial Production. If, within the methanolic extract an inhibitor was present as the previous results

implied, it could be expected that addition of this fraction to media would cause a suppression of fungal growth. Figure 28A in fact demonstrates this effect with mycelial growth of treatment Complete MS 0.5% being depressed to a level below that of Complete C 0.5% to approximately the same extent as mycelial growth of Complete S 0.5% was in excess of Complete C 0.5% (c.f. Figures 27A and 28A).

Growth in Complete WSt 0.5% is very similar to Complete MS 0.5% probably because of the absence of the water soluble, starch containing fraction in the wood.

(b) Percent dry weight loss. Actual weight loss in in Complete MS 0.5% and Complete WSt 0.5% is lower than Complete C 0.5% (Figure 28B). As can be seen from Figure 28C this is a function of mycelial growth which, when taken into account, causes the relative weight lost in treatments receiving extracts as a carbon source to be higher than Complete C 0.5%.

The higher relative weight loss in Complete MS 0.5% and Complete WSt 0.5% than in the comparable treatments Complete S 0.5% and Complete St 0.5%, may be explained in terms of the inadequacy of the carbon sources in the extractable fractions to act as a nutrient source

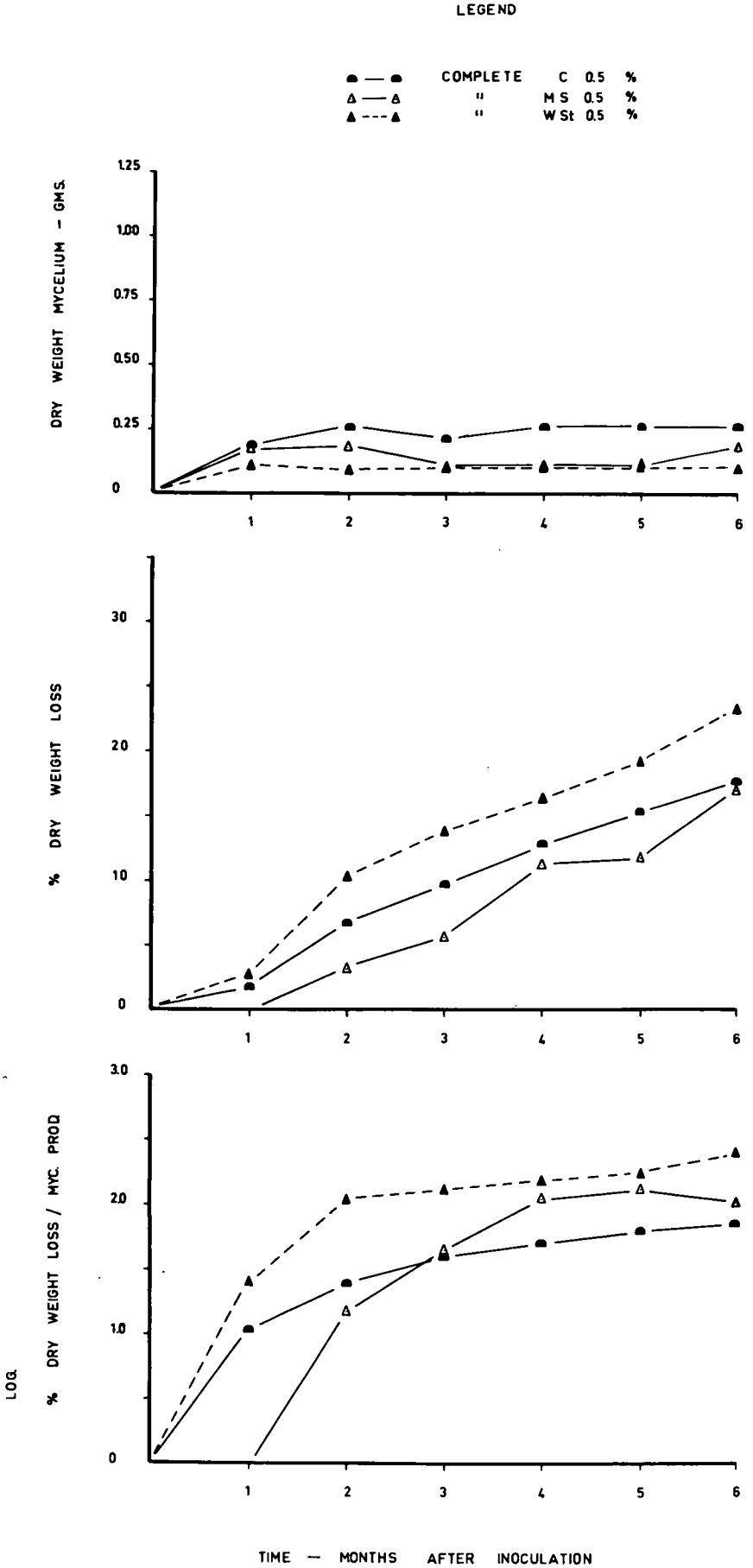
FIGURE 28

Experiment 4

28A (above) - Mycelial production of treatment Complete C 0.5% compared with treatments Complete MS 0.5% and Complete WSt 0.5%.

28B (centre) - Percent dry weight loss of Complete C 0.5% compared with Complete MS 0.5% and Complete WSt 0.5%.

28C (below) - Log. percent dry weight loss of Complete C 0.5% compared with treatments Complete MS 0.5% and Complete WSt 0.5%.


LEGEND
COMPLETE C 0.5 %
" M S 0.5 %
" W St 0.5 %
DRY WEIGHT MYCELIUM - GMS.
1.25
1.00
0.75
0.50
0.25
0
% DRY WEIGHT LOSS
30
20
10
0
LOG
% DRY WEIGHT LOSS / MYC. PROD.
3.0
2.0
1.0
0
1
2
3
4
5
6
TIME — MONTHS AFTER INOCULATION

for the fungus at the levels which were added. Hence although less mycelium was produced, wood blocks were attacked more vigorously to support the fungal growth present.

The overall effects of the treatments in this experiment are displayed in Figure 29A and 29B and Figure 30. In Figure 29A mycelial production for all treatments is shown for the six month duration of the experiment, and plotted in order of decreasing growth. Treatments receiving 2.0% sucrose in the media all induced a comparatively large quantity of growth. Treatment Complete S 0.5% demonstrates the effect of removing a probable inhibitor, contained within the methanolic fraction of the wood extract. Complete MS 0.5% shows ultimate suppression of fungal growth to a level below Complete C 0.5% and similar to Complete WSt 0.5%, while the lowest amount of growth is recorded in Complete H 0.5%.

Percent dry weight loss is shown for all treatments over the six month period of the experiment in Figure 29B. In treatments receiving 2.0% sucrose in the media the fungus has little effect on the wood for the first two to three months then decay increases slowly with time. Of the other treatments Complete S 0.5% and Complete St 0.5%

FIGURE 29

Experiment 4

29A (above) - Mycelial growth of all treatments included in experiment 4 plotted with respect to time in order of decreasing fungal growth.

29B (below) - Percent dry weight loss of all treatments included in experiment 4 plotted in order of decreasing fungal growth against time.

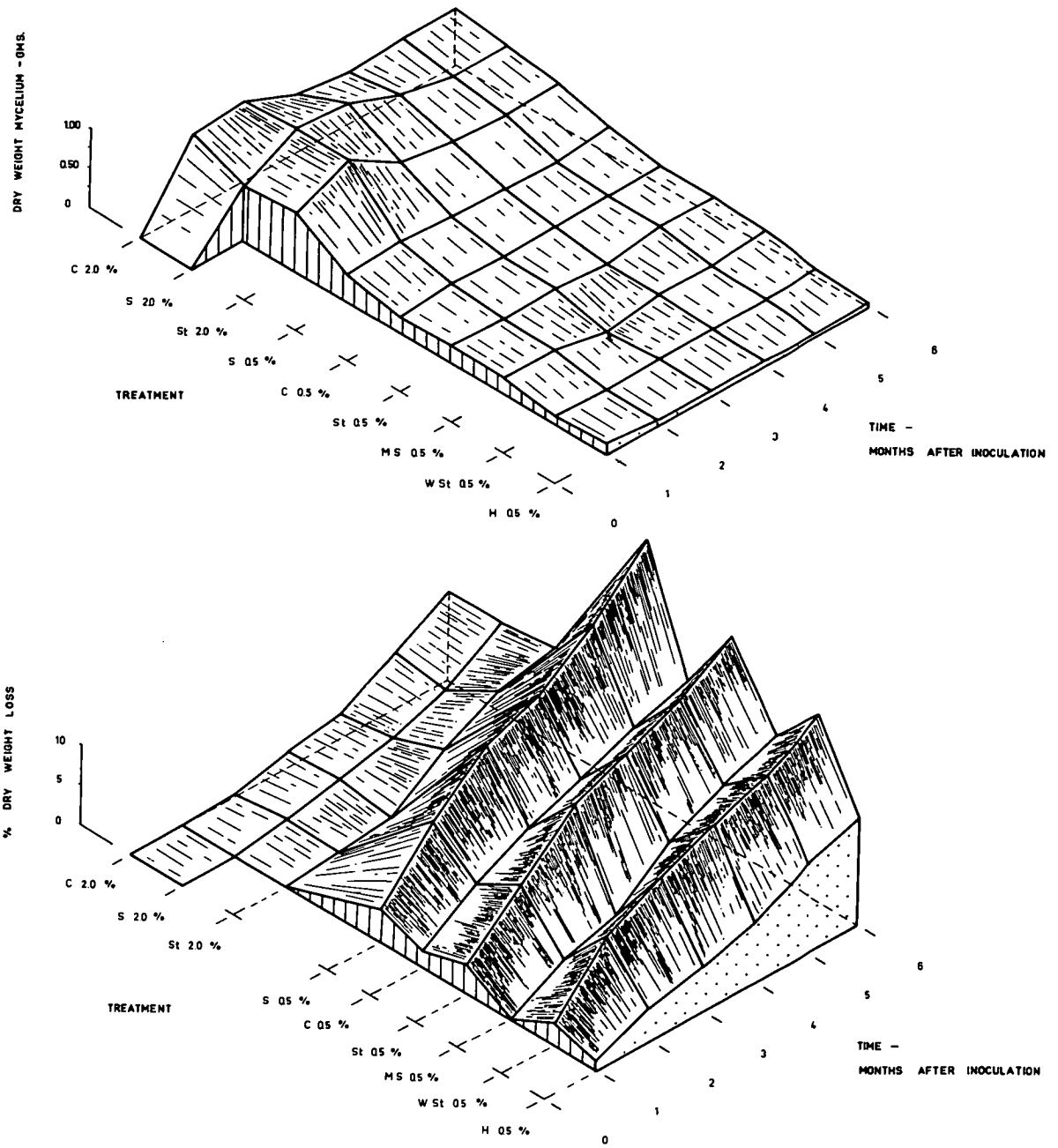
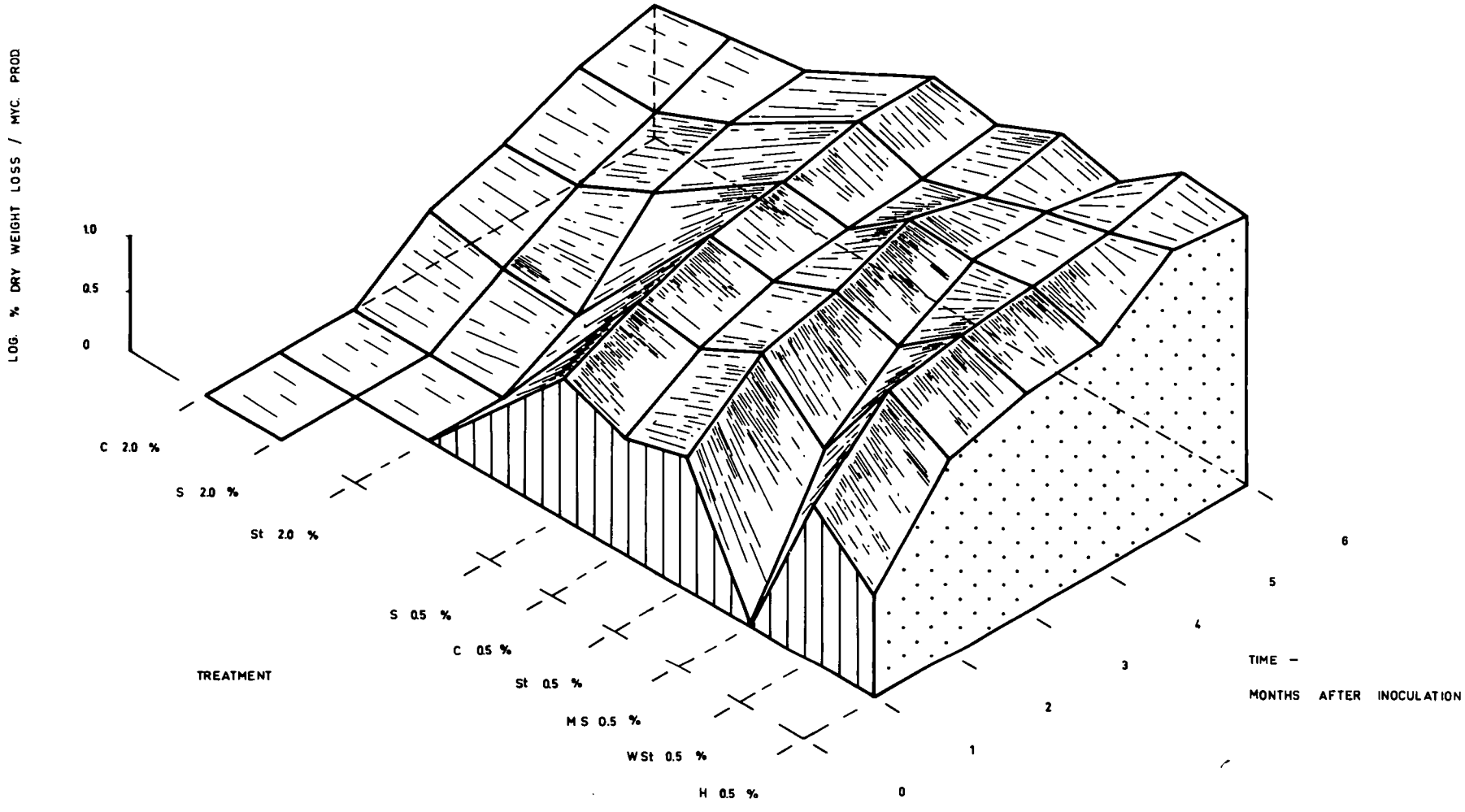

DRY WEIGHT MYCELIUM - DMS.
1.00
0.50
0
C 2.0 %
S 2.0 %
St 2.0 %
S 0.5 %
C 0.5 %
St 0.5 %
M S 0.5 %
W St 0.5 %
H 0.5 %
TREATMENT
0
1
2
3
4
5
6
TIME -
MONTHS AFTER INOCULATION
% DRY WEIGHT LOSS
10
5
0
C 2.0 %
S 2.0 %
St 2.0 %
S 0.5 %
C 0.5 %
St 0.5 %
M S 0.5 %
W St 0.5 %
H 0.5 %
TREATMENT
0
1
2
3
4
5
6
TIME -
MONTHS AFTER INOCULATION

FIGURE 30

Experiment 4

Log. percent dry weight loss/mycelial production in all treatments in experiment 4 plotted in order of decreasing mycelial production against time.


LOG. % DRY WEIGHT LOSS / MYC. PROD.
1.0
0.5
0
C 2.0 %
S 2.0 %
St 2.0 %
S 0.5 %
C 0.5 %
St 0.5 %
M S 0.5 %
W St 0.5 %
H 0.5 %
TREATMENT
0
1
2
3
4
5
6
TIME —
MONTHS AFTER INOCULATION

both display high and similar amounts of decay. Initially no decay occurs in Complete MS 0.5% due to the effect of the methanolic extract while in Complete WSt 0.5% although the fungus produces only a small amount of mycelium wood is attacked vigorously to sustain mycelial growth.

The two most striking features of Figure 30, where relative weight losses of each treatment are plotted against time, are (i) the absence of early decay in treatments receiving 2.0% sucrose despite the extraction of blocks and (ii) the temporary inhibitory effect of the methanolic extract in the treatment Complete MS 0.5%. Greatest relative amount of decay has occurred in treatments receiving the lower levels of sugar added to the media.

It is considered that the methanolic extraction of wood blocks in no way influenced the results obtained in this experiment. Mycelial growth of Complete S 2.0% was equivalent to that of Complete C 2.0% (Figure 26A). Complete S 0.5% in which blocks were extracted with methanol, produced a greater amount of mycelium than Complete C 0.5% where the blocks were untreated.

DISCUSSION

Experiment 1, 1A and 2 demonstrated a suppressing effect, in the presence of an easily available, soluble form of sugar, both to the amount of decay of wood by T. versicolor (Figures 17 and 20) and to the production of extracellular polyphenol oxidase (Figure 18). This suggests that in the presence of forms of sugar that can be readily utilized, the fungus will use these in preference to wood carbon.

The preceeding point was clearly demonstrated in experiment 3 when, with increasing media sugar levels, both actual and relative amounts of decay decreased (Figure 22). In addition enzyme production (experiment 3A) could be greatly suppressed if 3.0%, 2.0% or 1.5% sugar was present in the media compared with 1.0%, 0.5% or an absence of sugar (Figure 25).

It may be concluded from these results that if a continuous and high level of soluble carbohydrate was maintained and available to the fungus, wood decay could be reduced. However an anomally in this explanation is the large amount of mycelium produced under conditions of high sugar. This means that any fluctuation to a lower

sugar status (as would occur in the branches of apple trees through seasonal effects - Literature Review, Section IIA) would give rise to extensive and vigorous wood decay.

Experiment 4 confirmed a direct but perhaps superficial effect of sugars in relation to wood decay (Figures 29B and 30). It introduced the possibility of an inhibitor to fungal growth being associated with the methanolic extractable fraction in apple wood. This is seen (i) in Figure 27A where removal of the methanolic fraction resulted in higher levels of mycelial production (Complete S 0.5%) compared with mycelium produced when grown in the presence of unextracted blocks (Complete C 0.5%) and (ii) in Figure 28A suppression of mycelial growth when the methanolic fraction was added to the media at a concentration equivalent to 0.5% sucrose (Complete MS 0.5%).

CHAPTER 3

The Effect of Inoculating Apple Trees with *Trametes versicolor*.

I. Introduction

The work reported in this Chapter was conducted originally in an attempt,

(a) to detect the influence of age of the wood of trees inoculated on the establishment of the fungus and hence development of dieback;

(b) to ascertain if whole trees differed in susceptibility to attack by T. versicolor;

(c) to see if inoculation at different times during the year affected the establishment of T. versicolor in inoculated branches.

Following the results obtained from experiments 1, 2 and 3 - Chapter 2, it was decided to include some carbohydrate estimations of wood from inoculated trees.

II. Methods

A. Experimental Design

A supercomplete quasi-latin square design was used for the proposed investigations (see Appendix III A for details). On each of two commercial orchards 12 trees, forming 3 replicates of four trees, were selected. Wood of one-, two-, three-, four-, six- and eight years of age

was inoculated in the Spring (September), Summer (December), Autumn (March) and Winter (July) following the experimental design described in Appendix III A.

B. Method of inoculation and recording results

Cultures of T. versicolor were grown using Media 1 (p. 89). Randomally selected branches were pruned to the desired age and trimmed with a Stanley knife. A piece of mycelium of T. versicolor was cut from a previously inoculated culture and placed on the wound. To reduce the rate of drying of the inoculum, the wound and adjacent mycelium were covered with aluminium foil ("Comalco Alfoil").

At monthly intervals after inoculation the extent of external dieback symptoms was measured and recorded in mms. An inoculated piece of wood showing such symptoms is seen in Fig. 31.

Inoculations were commenced in September 1965 and completed in July 1966. Having recorded the extension of dieback symptoms for 12 months, each piece of inoculated wood was removed well below the extent of symptoms to ensure arrest of the spreading mycelium. The newly created wound was treated with an oil paint containing copper oxychloride to prevent further infection of the limb from

FIGURE 31

An inoculated branch of an apple tree displaying dieback symptoms following inoculation with *T. versicolor*.

natural, air borne inoculum.

All recordings were completed in July 1967.

C. Estimation of carbohydrate levels associated with inoculated wood

Carbohydrate estimations were carried out on the prunings from inoculated branches. It was not possible to analyse all prunings because of a lack of adequate amounts of material. Analyses were restricted to wood tissues and carried out only on December and March prunings. The method for carbohydrate analysis proposed by Priestley (1960) was adopted.

III. Results

Results from inoculation trials are presented in Appendix III B. Statistical analysis of the raw data was carried out excluding wood of one-year's age, which when inoculated did not appear to support growth of *T. versicolor*. This analysis is presented in Appendix III C.

Orchard 1 displays a significant difference in the extent of dieback between replicates, a significant replicate, treatment interaction plus a difference between treatments that are close to being significant at the 5%

level.

Using Duncan's multiple range test it can be seen (Addendix III C) that the July inoculations result in significantly higher amounts of dieback than the March inoculations. Although the July inoculations result in very much higher amounts of dieback than the December and September inoculations, the difference is not significant. Replicate 3 displayed significantly more dieback than Replicates 1 and 2 following the July inoculations. Within Replicate 3 it may also be seen that the July inoculations result in much more dieback than any of the other times of inoculation, these differences being significant at the 1% level.

Following statistical analysis of the data, Orchard 2 displayed significant differences between replications and treatments. It can be seen (Appendix III C) that the July inoculations have resulted in significantly higher amounts of dieback than that resulting from any other time of inoculation. Replicate 1, following the July inoculations produced a significantly higher amount of dieback than Replicate 2 and, although not statistically significant, higher also than Replicate 3.

Because of a significant difference between replicates, an error, on the part of the investigator was realized, in that each replicate within both orchards consisted of different varieties. Due to large differences between replicates, transformations on all data was necessary as well as sight alteration of the method of statistical analysis.

Analysis of transformed data is presented in Appendix III D. Here replicates are treated as varieties and as may be seen from Appendix III D in orchard 1, a significant difference exists between varieties, while in orchard 2 although no significant difference exists Duncan's multiple range test shows that dieback in variety 1 is significantly higher than either variety 2 or variety 3.

With regard to time of inoculation, although not significant, the July inoculations of orchard 1 result in a greater amount of dieback than that from inoculations at any other time. In orchard 2 the July inoculations are significantly higher than September and March inoculations. Following data transformations, the December inoculations are not significantly lower than July inoculations but a considerable difference does exist (Appendix III D). The

results also indicate (Appendix III, C and D) that apart from one-year old wood no differences existed in the effects of inoculation of woods of different ages.

The most important results from the trials are summarised in Tables 6, 7 and 8. Table 6 indicates the replicate and associated variety within each orchard and total amount of dieback resulting from inoculation. Table 7 contains means of total dieback for the different times of inoculation on the two orchards. Carbohydrate estimations of wood adjacent to that inoculated were performed at the time of inoculation. Table 8 records the results of the carbohydrate analysis of wood from the two orchards, following the December and March inoculations, together with the extent of dieback that subsequently developed.

Statistically significant differences do not exist in a number of areas of these inoculation trials. However, because the results have interesting implications with regard to varietal effect and time of inoculation, discussion will be undertaken appreciating,

(a) an error on the investigator's part in using different varieties as replicates.

(b) the need to increase the originally planned length of

TABLE 6

Replicate and associated variety in the two orchards showing the total dieback for each replicate

	Orchard 1			Orchard 2		
	Variety	Total Dieback (mms.)	Log (X+1) Transformed Total Dieback	Variety	Total Dieback (mms.)	Log (X+1) Transformed Total Dieback
Replicate 1	Delicious	94	9.333	Jonathan	532	24.398
Replicate 2	Sturmer	125	10.325	Delicious	202	14.755
Replicate 3	Jonathan	252	16.880	Crofton	306	19.059

TABLE 7

Treatment - (Time of inoculation) - Means

	Treatment	Actual Mean (mms)		Treatment	Transformed Mean - Log (X+1)	
Orchard 1	D (July)	13.9	s.d. 5%	D (July)	0.890	
	A (Sept.)	6.5		B (Dec.)	0.570	
	B (Dec.)	6.2		A (Sept.)	0.533	
	C (March)	4.7		C (March)	0.439	
Orchard 2	D (July)	31.1	s.d. 1%	D (July)	1.286	s.d. 5%
	B (Dec.)	18.0		B (Dec.)	0.958	
	A (Sept.)	11.5		C (March)	0.825	
	C (March)	8.6		A (Sept.)	0.810	

TABLE 8

Average sugar level and associated extent of dieback at two different times of noculation.

Treatment (Time of Inoculation)	% Soluble Sugar	% Starch	% Hemi-cellulose	Total Extractable Sugars	Total Dieback of all inoculated wood at time specified (mms.)
B (December)					
Orchard 1 (8)[x]	19.9	9.2	50.5	78.5	107
Orchard 2 (5)	9.2	3.1	33.9	46.3	262
C (March)					
Orchard 1 (8)	21.9	7.4	52.5	81.7	62
Orchard 2 (8)	15.2	5.7	51.7	72.7	141

x Figure in brackets indicates number of samples analysed to give average values for extractable sugars.

the trials from 12 to 18 months.

(c) that partly because of (a) and (b) statistically significant differences may not exist in all cases.

IV. Discussion

Varieties and associated dieback symptoms displayed after inoculation, are seen in Table 6. In both orchards Jonathan appeared to be far more susceptible following inoculation with T. versicolor than any of the other varieties. This result suggests that differing varietal resistance may be anticipated.

Evidence to support a proposal for an inhibitory factor contained within the methanolic extract of apple wood has already been mentioned (Chapter 2). It may be postulated that such an inhibitory factor could be associated with high sugar levels. Looking at Table 8, although varietal levels cannot be separated, it can be seen that in December, Orchard 1 has considerably higher soluble sugar levels than Orchard 2. Total dieback in Orchard 1 is less than half that of Orchard 2. Although in March the difference in sugar levels are not as accentuated, Orchard 1 still has the higher levels while dieback from inoculation is much lower.

The suggestion from this evidence is that for an effective inhibition of fungal destruction of apple branches, consistently high sugar levels are necessary. This idea is further supported by the results expressed in Table 7. Here it is seen that July inoculations (at which time reserve sugar levels in the wood may be expected to be at a maximum) result in the development of more extensive dieback symptoms than inoculations at any other time. This is consistent in both orchards but at a level twice as high in orchard 2 than in orchard 1. The higher reserve sugar levels anticipated in trees in July may have given rise to more efficient establishment of the fungus following inoculation. However the lower sugar status of experimental trees in orchard 2 resulted in the development of severe dieback symptoms compared with trees of orchard 1.

GENERAL SUMMARY AND CONCLUSIONS

Australian apple orchards are particularly susceptible to "Polystictus dieback", a disease associated with the invasion of tree limbs by Trametes versicolor. Previously reported associations between mineral element deficiencies and the disease were not confirmed in a field survey of Southern Tasmanian apple trees.

A number of plant diseases have been shown to be high sugar diseases or low sugar diseases, that is encouraged by high sugar or discouraged by high sugar (Horsfall and Dimond, 1957). Horsfall and Dimond (1957) considered that in the case of high sugar diseases one might expect the sugar to act directly in increasing the incidence of the disease. However with low sugar diseases it was suggested that they were correlated with a low "something else". In other words the low sugar status is merely an expression of a particular physiological state of the plant.

The data presented in this thesis indicate that T. versicolor attack of apple trees is a low sugar disease. It is quite conceivable that soluble sugars extractable in the methanolic fraction could be positively correlated with the possible inhibitor to the growth of the fungus, also contained within this fraction. Hence such an

inhibitor could be considered to be the "something else" mentioned by Horsfall and Dimond (1957). An inhibitor to the growth of wood rotting fungi and extractable from wood has been previously demonstrated. For example Da Costa and Rudman (1958) found that methanolic extracts from the decay resistant timber tallowwood (Eucalyptus microcorys) were highly toxic to tested wood rotting fungi. Osborne and Thrower (1964) suggested an inhibitory factor to the growth of fungi, present in the methanolic extract of timbers resistant to wood rotting fungi.

This being the case, in the presence of high soluble sugar levels in the wood of apple trees and an associated high level of inhibitor, the fungus would be subjected not only to inhibition of growth but also, because of the high sugar status, an inhibition of production of enzymes partly responsible for wood breakdown. It appears quite reasonable to explain differences in varietal susceptibility in terms of an inherent inability of some varieties, to accumulate reserve materials effectively and so have an inhibitory system capable of withstanding attack by T. versicolor.

Any factors then that lead to the inability of apple trees to effectively accumulate reserve carbohydrates, may be expected to render branches of trees so affected, more

susceptible to destruction by T. versicolor, should a suitable site of entry be available to inoculum of the fungus. Conditions reducing the nutrient absorbing potential of roots (drought, water-logging and mechanical root injury), overcropping, heavy pruning and mineral element deficiencies may all contribute to this effect.

A logical explanation may then be made for the appearance of "Polystictus dieback" in the increased susceptibility of trees to attack by T. versicolor being physiologically expressed in terms of a lower sugar status of branches.

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APPENDICIES

Appendix 1

APPENDIX 1

CHEMICAL ANALYSIS

Estimation of phosphorus, potassium and calcium in apple tree wood.

Solutions required

(1) Strong Acetate Buffer

272 gms. NaAc

440 mls. Glacial Acetic Acid

in 2 liters of distilled water.

(2) Weak Ammonium Acetate Buffer

7.7 gms. NH_4Ac (0.025N)

22.9 mls. Glacial Acetic Acid (0.1N)

in 4 liters of distilled water.

(3) Ammonium Molybdate

5% aqueous solution 50 gms. Ammonium molybdate dissolved in 1000 mls. of distilled water and stored in a dark, wax-lined bottle.

(4) Ascorbic Acid

1% in a weak acetate buffer (0.025N NaAc, 0.1N Acetic Acid)

(5) Standard Phosphate Solution

Stock solution containing 1 mgm. P/ml.

4.393 gms. KH_2PO_4/liter of distilled water.

(6) Standard Potassium Solution

Stock solution containing 1 mgm. K/ml.

1.909 gms. KCl/liter of distilled water

(7) Standard Calcium Solution

Stock containing 1 mgm. Ca/ml.

2.497 gms. $CaCO_3$ dissolved in concentrated HCl then diluted to 1 litre with distilled water.

(8) Concentrated Acid Mixture (for digestion)

Perchloric Acid ($HClO_4$) 70%

Sulphuric Acid (H_2SO_4) concentrated

Equipment

(1) One S30 hotplate for each aluminium digestion block (containing 24 holes each holding one test tube for digestion of wood samples).

(2) Bausch and Lomb Spectronic 20 Spectrophotometer for phosphate estimation.

(3) Eel Flame Photometer for potassium and calcium estimations.

(4) Glassware

(a) One 5 ml. capacity automatic pipitte with delivery tube shortened to hold an inverted two way stopcock, attached for the purpose of receiving constant sample and mixing it with 5 mls. of strong buffer contained in the above pipette.

(b) Three automatic burettes for quick delivery of acid, molybdic acid and ascorbic acid.

A stock of 6x$\frac{3}{4}$" test tubes - some graduated to 25 mls., and others matched for use in the Spectronic 20.

Method

To enable the estimation of phosphorus, potassium and calcium from one digest the following method was employed.

Initially three standard calibration curves were plotted from standard solutions of P, K and Ca. 0.1 gms. of previously ground and dried wood was weighed and placed in a test tube. 1 ml. of acid was added and the mixture digested for 6 hours. The digest was allowed to cool then made to 25 ml. using the NH_4Ac buffer. An aliquot of this was then taken and mixed with 5 mls. of the strong acetate buffer in a clean test tube. 1 ml. of ammonium molybdate was then added followed by 2 mls. of ascorbic acid. The colour was allowed to develop for 35 minutes, the intensity of which was measured at a wave length of 650 mu. Calcium and potassium were estimated directly from the remaining diluted digest.

Appendix 2

APPENDIX IIA

Regression equations for orchards involving the following variables.

X_7 = Degree of Infection X_4 = K, Ca ratio

X_1 = Phosphorus X_5 = P, K "

X_2 = Potassium X_6 = P, Ca "

X_3 = Calcium

Orchard Number

1. $X_7 = 22.7+290X_1-46.7X_2-17.8X_3+16.5X_4-56.3X_5-111.0X_6$
2. $X_7 = 20.0-79.1X_1+23.4X_2-22.2X_3-11.3X_4-20.2X_5+31.5X_6$
4. $X_7 = -8.05+75.9X_1-16.7X_2+17.7X_3-0.065X_4-10.8X_5+30.8X_6$
5. $X_7 = -11.9-46.8X_1-3.85X_2+20.0X_3+4.77X_4+17.0X_5+7.63X_6$
6. $X_7 = -37.7-361X_1+63.4X_2+33.6X_3-1.22X_4+137.0X_5+26.7X_6$
7. $X_7 = -16.13-226X_1+32.7X_2+13.5X_3-3.91X_4+66.2X_5+43.3X_6$
8. $X_7 = 9.53-33.7X_1-4.96X_2+4.960X_3-0.507X_4-17.3X_5+12.7X_6$
9. $X_7 = -20.6-13.0X_1-34.8X_2+61.0X_3+15.2X_4-0.748X_5-4.65X_6$
10. $X_7 = 49.1+197X_1-38.1X_2-8.19X_3-0.82X_4-170X_5-14.1X_6$
12. $X_7 = -26.2-107X_1+31.9X_2+1.02X_3-3.96X_4+198X_5-18.1X_6$
16. $X_7 = 19.7-186X_1+94.5X_2-93.5X_3-38.4X_4+167X_5+44.9X_6$
18. $X_7 = 23.9+373X_1-27.2X_2-82.0X_3+17.3X_4+27.2X_5-153X_6$
19. $X_7 = -0.974-95.2X_1+25.2X_2-7.5X_3-7.49X_4+3.11X_5+41.1X_6$
20. $X_7 = 10.0+6.27X_1-0.297X_2-3.73X_3-3.12X_4-30.8X_5+14.9X_6$
23. $X_7 = 12.2-49.7X_1+18.9X_2-8.05X_3-8.01X_4-9.18X_5+15.5X_6$
24. $X_7 = -107-709X_1+18.3X_2+228X_3-13.7X_4+25.2X_5+353X_6$
37. $X_7 = 34.1-214X_1+58.7X_2-29.7X_3-28.9X_4-32.7X_5+71.5X_6$
43. $X_7 = 4.17+300X_1-62.8X_2-1.78X_3+34.7X_4+22.9X_5-175X_6$

APPENDIX IIB

Values of "t" for regression coefficients.

(Corresponding to regression equations in Appendix IIA)

Orchard Nos.	N	X_1	X_2	X_3	X_4	X_5	X_6
1	26	0.935	2.290+	0.279	1.035	0.829	1.567
2	26	0.478	0.595	0.412	0.813	0.372	0.560
4	38	0.387	0.334	0.310	0.004	0.103	0.520
6	30	1.558	0.915	0.547	0.082	1.880	0.809
7	28	0.951	0.783	0.257	0.368	2.196+	0.692
8	26	0.314	0.260	0.134	0.043	0.319	0.262
9	44	0.278	2.315+	2.270+	3.114+	0.042	0.199
10	36	0.477	0.522	0.305	0.040	1.710	0.120
12	19	0.285	0.431	0.018	0.293	2.876+	0.437
18	20	1.260	0.518	1.153	1.708	0.232	1.391
20	26	0.087	0.040	0.364	2.180+	0.510	0.855

X_1 = Phosphorus

X_2 = Potassium

X_3 = Calcium

X_4 = K, Ca ratio

X_5 = P, K "

X_6 = P, Ca "

+ Values of "t" significant at 5% level.

APPENDIX C

Correlation Coefficient — Orchard No. 1

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.760	0.581	0.473	0.196	0.756	-0.206
K			0.286	0.847	-0.461	0.707	-0.163
Ca				-0.253	0.275	-0.084	-0.132
K/Ca					-0.564	0.792	-0.088
P/K						0.017	-0.071
P/Ca							-0.164
DI							1.000

Orchard No. 2

P		0.764	0.505	0.411	0.147	0.511	-0.178
K			0.435	0.717	-0.473	0.288	-0.118
Ca				-0.291	-0.020	-0.433	-0.120
K/Ca					-0.501	0.641	-0.058
P/K						0.272	-0.055
P/Ca							-0.056
DI							1.000

Orchard No. 4

P		0.698	0.543	0.371	0.192	0.654	0.356
K			0.269	0.833	-0.552	0.545	0.046
Ca				-0.280	0.226	-0.227	0.208
K/Ca					-0.684	0.650	-0.076
P/K						0.039	0.340
P/Ca							0.249
DI							1.000

Correlation Coefficients

Orchard No. 5

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.624	0.549	0.219	0.574	0.720	0.076
K			0.556	0.592	-0.269	0.252	0.039
Ca				-0.322	0.125	-0.126	-0.022
K/Ca					-0.372	0.469	0.164
P/K						0.588	0.075
P/Ca							0.242
DI							1.000

Orchard No. 6

P		0.443	0.495	-0.023	0.411	0.400	0.078
K			0.486	0.517	-0.614	-0.150	-0.023
Ca				-0.474	-0.066	-0.543	0.065
K/Ca					-0.504	0.426	-0.012
P/K						0.525	0.192
P/Ca							0.144
DI							1.000

Orchard No. 7

P		0.534	0.388	0.260	0.307	0.644	0.090
K			0.351	0.747	-0.609	0.200	-0.335
Ca				-0.326	-0.100	-0.434	-0.292
K/Ca					-0.539	0.510	-0.137
P/K						0.422	0.566
P/Ca							0.378
DI							1.000

Correlation Coefficients

Orchard No. 8

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.854	0.898	0.217	-0.398	-0.293	-0.446
K			0.776	0.585	-0.779	-0.296	-0.395
Ca				-0.032	-0.361	-0.646	-0.419
K/Ca					-0.791	0.378	-0.081
P/K						0.200	0.172
P/Ca							0.187
DI							1.000

Orchard No. 9

P		0.668	0.412	0.378	0.104	0.420	-0.282
K			0.620	0.613	-0.559	0.005	-0.037
Ca				-0.216	-0.308	-0.602	-0.092
K/Ca					-0.436	0.607	0.180
P/K						0.328	-0.289
P/Ca							-0.020
DI							1.000

Orchard No. 10

P		0.679	0.133	0.245	0.034	0.420	-0.134
K			-0.223	0.775	-0.686	0.631	0.109
Ca				-0.670	0.508	-0.736	-0.199
K/Ca					-0.843	0.875	0.197
P/K						-0.517	-0.335
P/Ca							0.084
DI							1.000

Correlation Coefficients

Orchard No. 12

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.533	-0.024	0.402	0.368	0.634	0.279
K			0.298	0.525	-0.568	0.099	-0.319
Ca				-0.601	-0.363	-0.548	0.092
K/Ca					-0.170	0.480	-0.370
P/K						0.531	0.655
P/Ca							0.156

Orchard No. 16

P		0.729	0.653	0.436	-0.231	0.185	-0.265
K			0.685	0.781	-0.812	-0.134	-0.626
Ca				0.094	-0.526	-0.469	-0.309
K/Ca					-0.684	0.219	-0.666
P/K						0.389	0.734
P/Ca							0.278

Orchard No. 18

P		0.653	0.731	0.056	0.322	0.462	0.051
K			0.606	0.610	-0.477	0.140	0.324
Ca				-0.190	0.027	-0.215	-0.006
K/Ca					-0.649	0.385	0.384
P/K						0.415	-0.359
P/Ca							-0.042

Correlation Coefficients

Orchard No. 19

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.222	0.442	-0.206	-0.036	0.073	0.464
K			-0.173	0.821	0.356	0.335	0.127
Ca				-0.671	-0.079	-0.849	-0.168
K/Ca					0.336	0.649	0.062
P/K						0.034	0.170
P/Ca							0.433
DI							1.000

Orchard No. 20

P		0.868	-0.028	0.620	-0.211	0.701	-0.055
K			-0.115	0.739	-0.615	0.713	-0.086
Ca				-0.554	0.125	-0.660	-0.115
K/Ca					-0.492	0.896	-0.221
P/K						-0.351	0.022
P/Ca							0.005
DI							1.000

Orchard No. 23

P		0.653	0.529	0.093	0.481	0.559	-0.054
K			0.372	0.578	-0.327	0.306	0.031
Ca				-0.494	0.200	-0.374	-0.006
K/Ca					-0.526	0.585	0.018
P/K						0.358	-0.099
P/Ca							-0.070

Correlation Coefficients

Orchard No. 24

	P	K	Ca	K/Ca	P/K	P/Ca	DI
P		0.691	0.725	0.492	0.154	0.584	0.100
K			0.920	0.902	-0.514	0.013	-0.251
Ca				0.503	-0.219	-0.132	-0.092
K/Ca					-0.673	0.088	-0.338
P/K						0.507	0.503
P/Ca							0.303

Orchard No. 37

P		0.705	0.449	0.507	0.136	0.492	-0.197
K			0.614	0.684	-0.489	0.020	0.150
Ca				-0.113	-0.256	-0.523	-0.025
K/Ca					-0.368	0.557	0.081
P/K						0.447	-0.380
P/Ca							-0.262

Orchard No. 43

P		0.804	0.400	0.544	-0.047	0.643	-0.172
K			0.341	0.802	-0.602	0.496	-0.012
Ca				-0.253	-0.126	-0.419	-0.175
K/Ca					-0.588	0.756	0.157
P/K						0.051	-0.217
P/Ca							-0.016

APPENDIX IID

TABLE 1

Average % D.W. of phosphorus, potassium and calcium in one year old wood of sampled trees in 18 orchards - analyses completed 20th April, 1965.

Orchard Number	Nos. of Samples N	Average % D.W.			Average Ratios of P, K, Ca		
		P	K	Ca	K/Ca	P/K	P/Ca
1	28	0.178	1.01	0.46	2.24	0.17	0.38
2	26	0.118	0.61	0.36	1.75	0.20	0.33
4	38	0.139	0.63	0.45	1.41	0.22	0.36
5	28	0.134	0.68	0.46	1.50	0.19	0.29
6	30	0.121	0.53	0.34	1.60	0.24	0.37
7	28	0.121	0.56	0.36	1.58	0.23	0.34
8	26	0.132	0.67	0.45	1.51	0.22	0.31
9	44	0.139	0.74	0.41	1.81	0.20	0.35
10	36	0.127	0.63	0.31	2.18	0.20	0.43
12	19	0.132	0.76	0.40	1.95	0.18	0.33
16	16	0.188	0.94	0.45	1.11	0.21	0.44
18	20	0.131	0.55	0.32	1.74	0.25	0.41
19	15	0.126	0.65	0.32	2.23	0.29	0.42
20	26	0.150	0.93	0.43	2.22	0.17	0.37
23	40	0.116	0.53	0.45	1.19	0.22	0.26
24	20	0.147	0.61	0.43	1.40	0.24	0.34
37	39	0.110	0.52	0.44	1.18	0.22	0.25
43	30	0.150	0.76	0.46	1.67	0.20	0.33

APPENDIX IIE

TABLE 2

Analysis of Variance Tables for Phosphorus, Potassium and Calcium.

(a) Phosphorus

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total	319	0.4701	
Orchard Mean	17	0.0862	0.00507
Within Orchards	302	0.3838	0.00127

$F = 3.992$ $F_{17,302,0.01} = 1.79$

i.e. there is a real difference between the mean phosphorus values of the 16 orchards

(b) Potassium

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total	319	22.924	
Orchard Mean	17	7.369	0.4334
Within Orchards	302	15.554	0.0515

$F = 8.416$ $F_{17,302,0.01} = 1.79$

i.e. a real difference between mean potassium levels of the 16 orchards.

Appendix IIE, Table 2 (continued)

(c) Calcium

Source of Variation	Degrees of Freedom	Sum of	Mean Square
Total	319	4.2212	
Orchard Mean	17	1.1995	0.07055
Within Orchard	302	3.0217	0.01000

$F = 7.05$ $F_{17,302,0.01} = 1.79$

i.e. there is a real difference between the mean calcium levels of the 16 orchards.

Appendix 3

APPENDIX IIIA

DESIGN TO TEST THE EFFECT OF INOCULATION OF THE FUNGUS TRAMETES (POLYSTICTUS) VERSICOLOR

(As supplied by Mr. A. Grassia - C.S.I.R.O., Stowell Avenue, Hobart).

It is desired to test the effect of the inoculation of the fungus Trametes (Polystictus) versicolor in the four different seasons to branches of apple trees of different ages.

A supercomplete quasi-latin square with four rows (trees), six columns (ages), four treatments (inoculations), and three replicates (in order to achieve balance), was suggested.

This quasi-latin square consists of a 4 x 4 latin square with two columns added. Therefore the same treatment appears in the same row once or twice.

The approach used for analysing balanced incomplete block designs is extended to this design.

The design and a systematic method to analyse it are shown in the attached Table A.

Considerations of a practical nature have led to a restriction in the randomization over ages. It is expected that branches of one year and two years of age have such a vigor that could give a consistent nil response to inoculation. The randomization was carried out in such a way that, if this happens, by discarding branches of age 1 and 2 we have a 4 x 4 latin square, properly randomized.

In the case of age 1 or 2, or both, giving a non-null response the complete analysis will introduce a small bias in the error, but this would be so negligible that we should not bother about it.

TABLE A

The design will be constructed by choosing at random three basic latin squares and randomizing, following the procedure of Fisher and Yates Tables. To these we add a pair of the following three possible basic sets of columns in which no combination of two treatments is repeated in the same set.

Set 1		Set 2		Set 3	
A	B	A	C	A	B
B	C	B	D	B	D
C	D	C	B	C	A
D	A	D	A	D	C

We choose at random one of these three sets, randomize columns and rows and add it to the first latin square. Then we choose another of the remaining two sets, randomize column and rows and add to the second latin square. The remaining set after randomizing column and rows will be added to the third latin square.

Appendix IIIA, Table A (continued)

Design - Orchard 1

		AGE					
Repl. I		1	2	3	4	6	8
Tree	1	B	A	D	A	C	B
	2	C	B	A	B	D	C
	3	D	C	B	C	A	D
	4	A	D	C	D	B	A
Repl. II							
Tree	1	C	D	B	C	D	A
	2	D	B	C	B	A	D
	3	A	C	A	D	C	B
	4	B	A	D	A	B	C
Repl. III							
Tree	1	B	D	C	B	A	D
	2	C	B	A	D	C	B
	3	D	A	B	A	D	C
	4	A	C	D	C	B	A

Take three plots, preferably contiguous, with four trees in each plot. Each plot forms a replicate.

Number the trees 1, 2, 3, 4 following the same order for all replicates. Then apply row 1 to tree 1, row 2 to tree 2, and so on. In the above plan a randomization has already been carried out.

Note that the four columns on the right hand side of each replicate form a complete latin square.

Appendix IIIA, Table A (continued)

Design - Orchard 2

AGE

Repl. I		1	2	3	4	6	8
Tree	1	A	B	B	D	A	C
	2	B	D	D	B	C	A
	3	C	A	A	C	B	D
	4	D	C	C	A	D	B
Repl. II							
Tree	1	B	A	C	B	A	D
	2	A	D	D	A	C	B
	3	C	B	A	D	B	C
	4	D	C	B	C	D	A
Repl. III							
Tree	1	A	C	D	A	C	B
	2	C	B	C	B	D	A
	3	B	D	A	C	B	D
	4	D	A	B	D	A	C

APPENDIX III B

External Symptoms Recorded 12 Months after Inoculation

For Treatments A = September B = December

C = March D = July

Orchard 1

Replicate 1

	Age 1		2		3		4		6		8	
Tree 1	0	(B)	15	(A)	5	(D)	0	(A)	0	(C)	0	(B)
2	0	(C)	18	(B)	0	(A)	8	(B)	10	(D)	0	(C)
3	0	(D)	0	(C)	10	(B)	0	(C)	0	(A)	0	(D)
4	0	(A)	10	(D)	0	(C)	8	(D)	10	(B)	0	(A)

Replicate 2

Tree 1	0	(C)	4	(D)	15	(B)	0	(C)	10	(D)	0	(A)
2	9	(D)	0	(B)	0	(C)	26	(B)	25	(A)	14	(D)
3	0	(A)	12	(C)	0	(A)	0	(D)	0	(C)	2	(B)
4	0	(B)	0	(A)	0	(D)	10	(A)	0	(B)	7	(C)

Replicate 3

Tree 1	0	(B)	10	(D)	15	(C)	0	(B)	5	(A)	42	(D)
2	0	(C)	13	(B)	15	(A)	44	(D)	0	(C)	5	(B)
3	0	(D)	5	(A)	0	(B)	0	(A)	45	(D)	15	(C)
4	0	(A)	4	(C)	5	(D)	9	(C)	0	(B)	20	(A)

Replicate 1 Delicious

Replicate 2 Sturmer

Replicate 3 Jonathan

Orchard 2

Replicate 1

	Age 1		2		3		4		6		8	
Tree 1	0	(A)	0	(B)	20	(B)	10	(D)	25	(A)	10	(C)
2	0	(B)	6	(D)	75	(D)	7	(B)	20	(C)	25	(A)
3	0	(C)	25	(A)	27	(A)	10	(C)	15	(B)	55	(D)
4	15	(D)	15	(C)	0	(C)	20	(A)	102	(D)	65	(B)

Replicate 2

Tree 1	0	(B)	0	(A)	5	(C)	10	(B)	15	(A)	0	(D)
2	0	(A)	15	(D)	5	(D)	0	(A)	10	(C)	15	(B)
3	20	(C)	20	(B)	7	(A)	10	(D)	50	(B)	10	(C)
4	0	(D)	0	(C)	0	(B)	0	(C)	30	(D)	0	(A)

Replicate 3

Tree 1	0	(A)	11	(C)	15	(D)	12	(A)	11	(C)	10	(B)
2	0	(C)	25	(B)	0	(C)	0	(B)	27	(D)	7	(A)
3	0	(B)	32	(D)	10	(A)	15	(C)	0	(B)	52	(D)
4	0	(D)	0	(A)	25	(B)	30	(D)	0	(A)	24	(C)

Replicate 1 Jonathan

Replicate 2 Delicious

Replicate 3 Crofton

APPENDIX III C

Statistical Analysis of Data Presented in Appendix III B.

(Including Ages 2-8 years)

Orchard 1

Grand Age Total	2	3	4	6	8	Total
	91	65	105	105	105	471

Grand Treatment Total	A	B	C	D	Total
	95	107	62	207	471

Treatment	Treatment Totals	Bt	Qi = 5T - Bt	ti = Qi/krE_f	Treatment Mean(adj.) = m + ti
A	95	573	-98	-1.361	6.489
B	107	649	-114	-1.583	6.267
C	62	533	-223	-3.097	4.753
D	207	600	+435	6.042	13.892
Total	471	2355	0		

m = 7.850

Bt = Gt. + total of blocks with t^{th} treatment twice

k = 5, r = 15, E_f = 0.96, rE_f = 14.4, krE_f = 72.0

k^2rE_f = 360

SST. (adj.) = $\frac{\text{Sum } Qi^2}{360}$

$\frac{Gt^2}{60} = \frac{221841}{60} = 3697.36$

$\frac{\text{Sum Replicate Totals}^2}{20} = \frac{87965}{20} = 4398.25$; SSR = 700.89

$\frac{\text{Sum Tree Totals}^2}{5} = \frac{24913}{5} = 4982.60$; SSB (within repl. unadj.) = 584.35

$\text{Sum Age Totals}^2 = \frac{45581}{12} = 3798.42$; SSA = 101.06

; $\text{SST(adj.)} = \frac{26155.4}{360}$

= 726.54

Sum Individuals^2 = 10, 503 ; SSt =6805.64

$\frac{\text{Sum Treatment Totals}^2}{15}$ = 447780 ; SST(unadj.)= 780.44

$\frac{\text{Replicate x Treatment Totals}^2}{5}$ = 6632.60; S.S. Repl. x Treat. = 1453.91

Analysis of Variance

Source of Variation	Degrees pf Freedom	Sum of Squares	Mean Square	Variance Ratio
Replicates	2	700.89	350.44	3.786 +
Trees (unadj. within repl.)	9	584.35	64.93	0.701 n.s.
Age	4	101.06	25.26	0.273 n.s.
Treatment (adj.)	3	726.54	242.18	2.617 n.s.
Treat. x Repl.	6	1453.91	242.31	2.618 +
Error	35	3238.89	92.53	
Total	59	6805.64		

$6\ ti^2 = 6.4263$ $6\ ti = 2.535$ (for treatments)

Duncan's Multiple Range Test - Treatments

Ranked Means

D	13.892	s.d. 5%
A	6.489	
B	6.267	
C	4.753	

5% k = 4, v = 35, q 4,35; 0.05 = 3.10

σ ti = 3.10 x 2.535 = 7.858

- Replicates (Varieties)

Means

	Replicate 1	Replicate 2	Replicate 3
A	3.0	7.0	9.0
B	9.2	8.6	3.6
C	0	3.8	8.6
		s.d. 1.0%	
D	6.6	5.6	29.2

s.d. 1.0%

σ ti^2 = 92.539/4.8; σ ti = 4.39
(for Replicates)

5% k = 3, v = 35, q 3,35; 0.05 = 3.01

σ ti = 3.01 x 4.39 = 13.21

1% k = 3, v = 35, q 3,35; 0.01 = 3.99

σ ti = 3.99 x 4.39 = 17.51

Orchard 2

Grand Age Total	2	3	4	6	8	Total
	149	189	124	305	273	1040

Grand Treatment Total	A	B	C	D	Total
	173	262	141	464	1040

Treatment	Treatment Totals	Bt	Qi = 5T - Bt	ti = Qi/krE_f	Treatment Mean(adj.) = m + ti
A	173	1281	-416	-5.777	11.556
B	262	1261	49	0.680	18.013
C	141	1331	-626	-8.694	8.639
D	464	1327	993	13.791	31.124
Total	1040	5200	0		

$m = 17.333$

$k = 5, \quad r = 15, \quad E_f = 0.96$

$rE_f = 14.4$

$krE_f = 72.0 \quad k^2rE_f = 360$

$$SST\ (adj.) = \frac{Sum\ Qi^2}{360}$$

$$\frac{Gt^2}{60} = \frac{1081600}{60} = 18026.666$$

$$\frac{Sum\ Replicate\ Totals^2}{20} = \frac{417464}{20} = 20873.200; \quad SSR = 2846.534$$

$\frac{\text{Sum Tree Totals}^2}{5} = \frac{118460}{5} = 23692.000$; SSB (within repl. unadj.) = 2818.800

$\frac{\text{Sum Age Totals}^2}{12} = \frac{240852}{12} = 20071.000$; S.S.A. = 2044.334

$$SST = \frac{155338.2}{360}$$

$$= 4314.950$$

$\text{Sum Individuals}^2 = 41,160$; SSt = 23,133.334

$\frac{\text{Sum Treatment Totals}^2}{15} = \frac{333750}{15} = 22250.000$; SST = 4223.334

$\frac{\text{Replicate x Treatment Totals}^2}{5} = \frac{137094}{5} = 27418.800$;

SS Treat. x Repl. = 2322.266

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	
Replicates	2	2846.534	1423.267	5.669	++
Trees (unadj. within repl.)	9	2818.800	313.200	1.247	n.s.
Age	4	2044.334	511.083	2.036	n.s.
Treatment(adj.)	3	4314.950	1438.316	5.729	++
Treat. x Repl.	6	2322.266	387.044	1.542	n.s.
Error	35	8786.450	251.041		
Total	59	23133.334			

$\sigma\ ti^2 = 17.4334$; $\sigma\ ti = 4.175$

(for treatments)

Duncans Multiple Range Test - Treatments

Ranked Means

D	31.124	s.d. 5%	s.d. 1%
B	18.013		
A	11.556		
C	8.639		

5% q 4, 35; 0.05 σ ti = 4.175 x 3.10 = 12.942

1% q 4, 35; 0.01 σ ti = 4.175 x 4.10 = 17.117

- Variety

Means

	Replicate 1	Replicate 2	Replicate 3
A	24.4	4.4	5.8
B	21.4	19.0	12.0
C	11.0	5.0	12.2
D	49.6	12.0	31.2

s.d. 5% (A–B, B–C, C–D)

s.d. 5% (A–C, B–D)

s.d. 5% (A–D)

s.d. 5% (A–B, B–C, C–D)

σ ti^2 = 52.3002 σ ti = 7.232

(for Replicates)

5% q 3, 35; 0.05 σti = 7.232 x 3.01 = 21.768

1% q 3, 35; 0.05 σ ti = 7.232 x 4.01 = 28.855

APPENDIX III D

Statistical Analysis for Data Presented in Appendix IIIB after Log (x+1) Transformations (Including Ages 2-8 years)

Orchard 1

Grand Age Total	2	3	4	6	8	Total
	9.001	6.209	7.033	6.978	7.317	36.538

Grand Treatment Total	A	B	C	D	Total
	7.742	9.175	6.124	13.497	36.538

Treatment	Treatment Totals	Bt	Qi = 5T - Bt	ti = Qi/krE_f	Treatment Mean(adj.) = m + ti
A	7.742	44.108	-5.398	-0.075	0.533
B	9.175	48.619	-2.744	-0.038	0.570
C	6.124	42.793	-12.173	-0.169	0.439
D	13.497	47.174	20.315	0.282	0.890
Total	36.538	182.694	0		

$m = 0.608$

Bt = Gt + total of blocks with t^{th} treatment twice.

$k = 5$, $r = 15$, $E_f = 0.96$

$rE_f = 14.4$, $krE_f = 72.0$

$k^2rE_f = 360.$

$$\text{SST (adj.)} = \frac{\text{Sum } Qi^2}{360}$$

$$\frac{Gt^2}{60} = \frac{13350.254}{60} = 22.2504$$

$$\frac{\text{Sum Replicate Totals}^2}{20} = \frac{478.644914}{20} = 23.9322;\ \text{SSR} = 1.6818$$

$$\frac{\text{Sum Tree Totals}^2}{5} = \frac{127.820792}{5} = 25.5641;\ \text{SSB (within repl. unadjusted)} = 1.6319$$

$$\frac{\text{Sum Age Totals}^2}{12} = \frac{271.263744}{12} = 22.6053;\ \text{SSA} = 0.3549;$$

$$\text{SST (adj.)} = \frac{597.549094}{360} = 1.6598$$

$$\text{Sum Individuals}^2 = 42.095852;\quad \text{SSt} = 19.8454$$

$$\frac{\text{Sum Treatment Totals}^2}{15} = \frac{363.791574}{15} = 24.2527;$$

$$\text{SST (unadj.)} = 2.0023$$

$$\frac{\text{Repl. x Treat. Totals}^2}{5} = \frac{144.863052}{5} = 28.9726;$$

$$\text{SS Repl. x Treat.} = 3.0381$$

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Varieties	2	1.6818	0.8409	5.868 +
Trees (within variety adj.)	9	1.2894	0.1433	
Total (a)	11	2.9712		
Treatment (unadj.)	3	2.0023	0.6674	
Trees (within unadj.)	+ 9	+ 1.6319	+ 0.1813	0.5529 n.s.
Treatment (adj.)	3	1.6598	0.5533	1.6874 n.s.
Age	4	0.3549	0.0887	0.2705 n.s.
Variety x Treat.	6	3.0381	0.5064	1.5444 n.s.
Error (b)	35	11.4789	0.3279	
Total	59	19.8454		

+ Not Included in Total.

Duncans Multiple Range Test - Treatments

Ranked Means

D	0.890
B	0.570
A	0.533
C	0.439

5% k = 4, v = 35, q 4, 35; 0.05 = 3.10

$\sigma ti^2 = 0.022770$; $\sigma ti = 0.151$

$\sigma ti = 0.151 \times 3.10 = 0.468$

No sig. diff. at 5%.

- Variety

Ranked Means

V_3	0.844
V_2	0.516
V_1	0.466

$\sigma ti^2 = 0.1433/48$; $\sigma ti = 0.1727$

5% q 3, 9; 0.05 = 3.34

$\sigma ti = 0.1727 \times 3.34 = 0.576$

No Sig. Diff. at 5%.

Orchard 2

Grand Age Totals	2	3	4	6	8	Total
	10.002	10.769	10.184	13,923	13.334	58.212

Grand Treatment Totals	A	B	C	D	Total
	12.179	14.393	12.228	19.412	58.212

Treatment	Treatment Total	Bt	Qi = 5t - Bt	ti = Qi/krE_f	Treatment Mean(adj.) = m + ti
A	12.179	72.380	-11.485	-0.1595	0.8107
B	14.393	72.810	- 0.845	-0.0117	0.9585
C	12.228	71.578	-10.438	-0.1449	0.8253
D	19.412	74.292	22.768	0.3162	1.2864
Total	58.212	291.060			

$$m = 0.9702$$

$k = 5, \quad r = 15, \quad E_f = 0.96$

$rE_f = 14.4$

$krE_f = 72.0; \quad k^2rE_f = 360$

$$SST\ (adj.) = \frac{\text{Sum } Qi^2}{360}$$

$$\frac{Gt^2}{60} = \frac{3388.636944}{60} = 56.4772$$

$$\frac{\text{Sum Replicate Totals}^2}{20} = \frac{1176.217910}{20} = 58.8108;$$

SSR = 2.3336

$$\frac{\text{Sum Tree Totals}^2}{5} = \frac{309.685024}{5} = 61.9370;$$

SSB (within replicate unadj.) = 3.1262

$$\frac{\text{Sum Age Totals}^2}{12} = \frac{691.370706}{12} = 57.6142; \quad \text{SSA} = 1.1370$$

$$\text{SST (adj.)} = \frac{759.952918}{360}$$

$$= 2.1109$$

$$\text{Sum Individuals}^2 = 77.534176 \qquad \text{SSt} = 21.0569$$

$$\frac{\text{Sum Treatment Totals}^2}{15} = 881.836218 = 58.7890;$$

SST (unadj.) = 2.3118

$$\frac{\text{Repl. x Treat. Totals}^2}{5} = 63.3526; \quad \text{Repl. x Treat.} = 2.2300$$

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Varieties	2	2.3366	1.1668	3.590
Trees (within var. adj.)	9	2.9253	0.3250	
Total (a)	11	5.2589		
Treatment (unadj.)	3	2.3118	0.7706	
Trees (within unadj.) +	9	3.1262	0.3474	1.2016 n.s.
Treatments (adj.) +	3	2.1109	0.7036	2.4337 n.s.
Age	4	1.1370	0.2842	0.9830 n.s.
Treat. x Variety	6	2.8687	0.3717	1.2857 n.s.
Error (b)	35	10.1192	0.2891	
Total	59	21.0569		

+ Not included in total.

$\delta\ ti^2 = 0.020090; \quad \delta\ ti = 0.141$

Duncan's Multiple Range Test - Treatments

Ranked Means

D	1.2864	s.d. 5%
B	0.9585	
C	0.8253	
A	0.8107	

5% q 4, 35; 0.05 = 3.10

σ ti = 3.10 x 0.141 = 0.437

- Variety

Ranked Means

V_1	1.219
V_3	0.952
V_2	0.737

5% q 3, 9 = 3.34

σ ti = 0.261 x 3.34 = 0.871

No Sig. Diff. at 5% level.